

# ADVANCES IN BOTANICAL RESEARCH

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VOLUME SIXTY FIVE

# ADVANCES IN BOTANICAL RESEARCH

## Genomics of Cyanobacteria

Volume Editors

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## PREFACE

In this book entitled ‘Genomics of Cyanobacteria’, a team of internationally renowned researchers expose the most up-to-date knowledge on cyanobacteria, the fascinating microorganisms with great evolutionary, ecological and biotechnological importance, which are logically receiving a growing attention in basic and applied researches. This book emphasizes the crucial importance of functional genomics in model cyanobacteria to characterize relevant gene products, and of comparative genomics analysis of various strains inhabiting diverse biotopes for a better understanding of the adaptation of cyanobacteria to natural environments. Even if you have no previous background in the subject, the book’s clear language and illustrations tell you what you need to know about cyanobacteria. It also highlights important directions for future researches aiming at better understanding cyanobacteria, in the prospect of turning their valuable biotechnological potentials into industrial realities.

Cyanobacteria, formerly named ‘blue-green algae’ are the only known prokaryotes capable of oxygenic photosynthesis. They are regarded as being among the oldest life forms on earth (~2.5 billion years); the producers of the Earth’s oxygenic atmosphere; and the progenitor of plant chloroplasts. Contemporary cyanobacteria exhibit a remarkable adaptation success in colonizing a wide range of biotopes (fresh, brackish and marine waters, and soils including deserts). Consistently, the genetic, genomic, metabolic and morphological diversity of cyanobacteria (Gram-negative prokaryotes) rival that seen among the totality of other (Gram-negative plus Gram-positive) eubacteria. Hence, cyanobacteria are attractive model systems to study these processes. The hardiness of cyanobacteria is due to their efficient photosynthesis that uses nature’s most abundant resources, solar energy, water, CO<sub>2</sub> and mineral nutrients, to produce a large part of the atmospheric oxygen and organic assimilates which sustain the biosphere. Cyanobacteria convert captured solar energy into biomass in the field at greater efficiencies (3–9%) than terrestrial plants (0.25–3%), and they tolerate higher CO<sub>2</sub> content in gas streams than higher plants. On a global scale, cyanobacteria fix an estimated 25 Giga tons of carbon from CO<sub>2</sub> per year into energy dense biomass. Consequently, in addition to being important producers of biomass for the food chain and of natural products with interesting biological activities, cyanobacteria are regarded as promising “low-cost” cell factories for the carbon-neutral production of renewable biofuels due to their simple nutritional

requirements, their metabolic robustness and plasticity, and the powerful genetics of some model strains. All these potentials benefit from the capacity of cyanobacteria to grow in a variety of locations, enabling industrial productions to be performed near the sites of use, to reduce transportation costs. Cyanobacteria are also fascinating in exhibiting a wide morphological diversity (unicellular, multicellular, filamentous, spherical or cylindrical shapes). Many multicellular cyanobacteria differentiate specialized cells, heterocysts and akinetes, for growth and survival under adverse conditions, and some strains can also establish symbioses with other organisms (fungi, sponges, bryophytes, gymnosperms, angiosperm, and the water fern *Azolla filiculoides*).

Up to now, the genome of more than 60 cyanobacteria living in diverse habitats has been fully sequenced, in the frame of meta-genomic analyses. These genomes ranging from 1.44 to 9.05 Mb in size (from 1200 to 8500 genes) comprise one circular chromosome, and in one case one linear chromosome too. While most marine cyanobacteria are monoploid or diploid, in harbouring, respectively, one or two copies of their chromosome per cell, non-marine strains are polyploid in propagating about 10 copies of their chromosome per cell. In addition to the chromosome, many cyanobacteria harbour also a few plasmids, some of which being up to several hundred kilobases in size. The comparison of the sequenced genomes is being used to determine which genes are present in any particular cyanobacterium inhabiting any particular biotope, and which ones are absent. In turn, these data serve for genome-based reconstruction of the whole metabolism of a few model cyanobacteria, as well as for reconstructions of genome evolution.

The eight chapters of this book, written by expert scientists from various countries, share with researchers and students, the most up-to-date knowledge on the fascinating biology and genomics of cyanobacteria, as follows. In the first chapter, Cheryl Kerfeld and Diana Kirilovsky report on the mechanisms used by cyanobacteria to protect themselves against an excess of light fluence. The conversion of solar light into chemical energy by plants and cyanobacteria is essential to life on earth. These organisms carry out oxygenic photosynthesis using two macromolecular assemblies known as Photosystem I and Photosystem II linked by an electron transport chain. When the amount of light energy exceeds the capacity of these organisms' photosynthetic apparatus to harness it, the photosynthetic electron transport chain becomes stalled in a reduced state and reactive oxygen species (ROS) are formed which lead to severe cell damages. Nutrient starvation and low CO<sub>2</sub> conditions predispose photosynthetic organisms to this threat at even relatively low irradiance. Cyanobacteria have evolved a key

and rapid photoprotective mechanisms to cope with abrupt and fluctuating changes in the quality and intensity of light in decreasing the effective size of the Photosystem II antenna. Light activates a soluble orange carotenoid protein (OCP), which interacts with the light-harvesting antenna (phycobilisomes) to increase thermal dissipation of absorbed energy, resulting in a decrease of energy arriving at the reaction centers. The increase of thermal dissipation also causes a decrease of the yield of phycobilisome fluorescence creating a non-photochemical fluorescence quenching. This chapter presents the emerging understanding of the OCP-mediated photoprotective mechanism, within the context of new genomic informations.

Besides light, the availability of water and the amount of dissolved ions (total salinity) are important environmental factors determining the occurrence of cyanobacteria in specific water and terrestrial environments. Because total salinity and water amount are closely linked, e.g. during desiccation the amount of water is decreasing in parallel with the increase in total salt concentration, it is not surprising that acclimation toward drought and high salinity employs overlapping mechanisms. In both cases, the maintenance of water and turgor pressure inside the cell is one of the central issues during the acclimation. Because water uptake is a passive process following the water potential gradient, growing cells need to establish a low water potential inside the cell relative to the surrounding medium. This is achieved by regulating the cellular osmotic potential via varying amounts of low molecular compounds. The main difference between pure water, or osmotic, stress and salt stress is the additional direct ion effects on metabolic activities in the latter case. In nature, the large variations in the amount and composition of inorganic salts clearly affect the distribution of cyanobacteria. Additional to the problem that high total ion contents generally make it difficult to maintain water and turgor inside the cell, many ions are toxic for living cells. This direct toxicity is not only true for heavy metals, but for any ion at non-physiological high-cellular concentrations. Among cyanobacterial strains, three main salt tolerance groups can be distinguished: low and moderate halotolerant cyanobacteria as well as hypersaline strains. Regardless of the final salt resistance, all cyanobacteria apply two basic strategies for a successful acclimation to enhanced salt concentrations: accumulation of compatible solutes combined with active export of toxic ions, particularly  $\text{Na}^+$  and  $\text{Cl}^-$ . The second chapter by Martin Hagemann reviews the existing mechanistic and genomic knowledges about cyanobacterial salt acclimation, an important process for future biotechnological applications, which will be performed preferentially in saline waters.

The third chapter by the groups of Nir Keren and Enrico Schleiff describes the processes used by cyanobacteria to provide large amounts of the essential micronutrient iron (Fe) to their Fe-rich photosynthetic apparatus, and a wealth of other iron-requiring enzymes crucial to cell metabolism. Although Fe is the fourth most plentiful element in the Earth's crust, it is frequently a growth-limiting nutrient in large regions of the ocean and in many freshwater environments. This is due to both Fe concentration and chemistry. In aqueous solutions, iron has two environmentally relevant oxidation states: Fe(II) and Fe(III). Prior to the evolution of oxygenic photosynthesis, reducing environmental conditions resulted in iron existing primarily in its reduced form, Fe(II). Ferrous ions Fe(II) are relatively soluble and thereby readily bioavailable at the circumneutral pH range. Upon the evolution of oxygenic photosynthesis, molecular oxygen build up in the atmosphere led to oxygenation of aquatic environments where Fe(II) species rapidly oxidized to Fe(III). Ferric ions Fe(III) are poorly soluble at the circumneutral pH range and precipitate out of solution as ferric oxyhydroxides which are not considered bioavailable. Thus, cyanobacteria living in diverse and highly variable environments utilize multiple strategies to maintain iron levels within a desired range. Depending on the chemistry and environmental bioavailability of Fe species, these processes include (i) synthesis, export and re-import of powerful ferric ion chelators called siderophores; (ii) dedicated energy-consuming uptake systems; (iii) sequestration of temporary extra Fe atoms in storage proteins (ferritins); and (iv) degradation of abundant iron-rich non-essential proteins in response to iron starvation to release Fe atoms for subsequent incorporation into crucial iron-requiring metabolic enzymes.

These complex processes require a tight coordination with the homeostasis of all metal ions (Mg, Mn, Zn, Cu, etc.) that serve as cofactors of the wealth of metalloenzymes operating in cell metabolism, as occurs in most biological organisms. The fourth chapter by the group of Maria Fillat reviews the major families of metalloregulators in cyanobacteria. The major metal regulator system is composed of Fur-type regulators and anti-sense RNA, which control the expression of a wealth of metal-regulated genes. These metal-sensing proteins, which harbour metal-binding and DNA-binding domains, are usually allosteric proteins. Their reversible interaction with the regulatory metal drives conformational changes that affect DNA binding and the subsequent regulation of various genes involved in various processes. These findings reveal interesting connections between metabolic networks and interactivity between major regulons. They provide a better

understanding of cyanobacterial physiology including maintenance of metal homeostasis in a highly variable environment, and of the strategies to deal with different stress-generated cell damages, as follows.

Because of their lifestyle, cyanobacteria are continuously challenged with toxic ROS present in our oxygenic atmosphere (ozone,  $O_3$ ), or generated by the metal-requiring cellular processes photosynthesis, respiration and cell metabolism. These oxidative agents are namely: singlet oxygen ( $^1O_2$ ), the superoxide anion ( $O_2^{\cdot-}$ ), hydrogen peroxide ( $H_2O_2$ ), and the hydroxyl radical ( $OH^{\cdot}$ ). Among other ROS-generated damages, cysteines can be oxidized to form sulfenic acid ( $-SOH$ ) and disulfide ( $-S-S-$ ) by a two-electrons transition; sulfinic acid ( $-SO_2H$ ) by a four-electrons transition; and eventually sulfonic acid ( $-SO_3H$ ) by a six-electrons transition. Two types of disulfide can be distinguished considering whether they link two cysteinyl residues, from the same or different proteins (intra- or inter-molecular disulfide bridges); or from a protein and a molecule of the antioxidant tripeptide glutathione (glutathione-protein mix disulfide, also termed glutathionylation). These sulfur switches can provide an important and flexible means of reversibly controlling protein function. Glutathionylation is regarded as a transient protection of critical cysteines against irreversible oxidation (sulfinic and sulfonic acids) during oxidative stress, and as a post-translational regulatory modification.

The ROS oxidants can be detoxified by various metabolites (ascorbate, carotenoids, glutathione, vitamins, etc.) and several enzymes, such as the superoxide dismutase (SOD), catalase and peroxidase, which sequentially convert the superoxide anion to hydrogen peroxide (SOD) and then to water (catalase and peroxidase). By contrast, the protein disulfides and glutathione-protein mix disulfides are repaired by thioredoxins and glutaredoxins. If and when the oxidants outnumber the antioxidants, the resulting oxidative stress can lead to cell death. In addition, the ROS species, more particularly  $H_2O_2$ , can also operate in signalling, which is an important physiological process. Indeed,  $H_2O_2$  possesses the required properties to be a secondary messenger in being enzymatically produced and degraded by the SOD and catalase enzymes, respectively. Furthermore,  $H_2O_2$  oxidizes protein thiols in disulfides, which can be reduced back to thiols are thereby relevant as thiol redox switches for signalling. The fifth chapter by the group of Corinne Cassier-Chauvat and Franck Chauvat reviews the variety of the processes used by cyanobacteria to protect themselves against oxidative stress, emphasizing on glutathione and the wealth of glutathione-dependent enzymes, because they have been well conserved during evolution. We also

report on what can be inferred in this field by mining the information provided by 40 sequenced genomes of morphologically and physiologically diverse cyanobacteria.

Another important aspect is that cyanobacteria produce a wide range of secondary metabolites with diverse chemical structures and biological activities (vitamins, sunscreens, antibiotics, anti-cancer, toxins, etc.). For instance, at least 800 different secondary metabolites have been identified in marine cyanobacteria so far, a number likely representing a small fraction of the natural product repertoire. The functions of most of these secondary metabolites are usually unknown, but it is assumed that their production gives some advantage to the producers in complex ecosystems. It has also been proposed that these molecules might be communication molecules although there is no firm experimental data on this issue. Some of the cyanobacterial secondary metabolites appeared to be cytotoxic, neurotoxic or dermatotoxic to animals and/or humans. In the past 10 years, many biosyntheses of cyanobacterial secondary metabolites have been deciphered, at the genetic and biochemical level. Thanks to the advent of genomic data on cyanobacterial genomes and to new powerful bioinformatic tools, about 30 clusters of genes responsible for the production of cyanobacterial secondary metabolites have been identified, including the cyanotoxins: microcystin, cylindrospermopsin, saxitoxin and anatoxin-a. Almost all cyanobacterial secondary metabolites are the products of polyketide synthases, non-ribosomal peptide synthases or hybrid thereof. However, ribosomal peptides are also produced by cyanobacteria, like the cyanobactins and recent genome mining data suggest that these metabolites are more represented than first thought in cyanobacteria. The sixth chapter by the group of Annick Mejean and Olivier Ploux gives an overview of the connections between cyanobacterial secondary metabolites and their biosynthetic genes, with emphasis on the most significant cases like cyanotoxins, sunscreens, alkanes and terpenes.

In addition to secondary metabolites, many cyanobacteria produce extracellular polymeric substances (EPSs), mainly composed of polysaccharides, which can remain associated to the cell or be released into the surrounding environment. The particular characteristics of these EPS, such as the presence of two different uronic acids, sulphate groups and high number of different monosaccharides (up to 13), makes them very promising for biotechnological applications. Despite the increasing interest in these polymers, the information about their biosynthetic pathways is still limited. Studies performed in other bacteria revealed that the mechanisms of EPS

assembly and export are relatively conserved, and require the involvement of polysaccharide copolymerase and outer membrane polysaccharide export proteins. In cyanobacteria, the genes encoding these proteins occur in multiple copies, scattered throughout the genome, either isolated or in small clusters. In addition, it is also necessary to identify other genes that may be related to this process, understand their genomic distribution, and reconstruct their evolutionary history. The data, reviewed in the seventh chapter by the groups of Roberto De Philippis and Paula Tamagnini, provide a first insight on the phylogenetic history of the EPS-related genes, and constitute a robust basis for subsequent studies aiming at optimizing EPS production in cyanobacteria.

Last, but not least of their fascinating capabilities, many cyanobacteria display multicellularity and cell differentiation. These cyanobacteria grow as chains (filaments) of contiguous, photosynthetically active, vegetative cells that divide actively under nitrogen-replete conditions (in the presence of nitrate and ammonium). When challenged by the absence of combined nitrogen, some cells in the filaments differentiate into heterocysts, the non-dividing, photosynthetically inactive, cells that perform the fixation of atmospheric (inorganic) nitrogen ( $N_2$ ). These thick-walled heterocyst cells provide a micro-oxic environment for the oxygen-sensitive nitrogenase enzyme to function and perform nitrogen fixation. In the developed multicellular filament bearing the two cell types, vegetative cells and heterocysts exchange metabolites, with heterocysts providing vegetative cells with fixed nitrogen, and vegetative cells providing heterocysts with photosynthates. In the eighth chapter, the group of Antonia Herrero and Enrique Flores summarizes the biochemical and morphological properties of the heterocysts to then focus on the program of gene expression that supports the process of differentiation and its regulation. These authors first describe the special envelope and metabolism that makes the heterocyst micro-oxic, to concentrate then on the regulation of gene expression during the process of differentiation. Heterocyst differentiation starts as a response to a persistent high-cellular carbon-to-nitrogen balance signalled by the level of the 2-oxoglutarate metabolite. In turn, this results in activation of the global transcriptional regulator NtcA followed by increased expression, mainly localized to differentiating cells, of the genes *ntcA* and *hetR*, which encodes HetR, the differentiation-specific transcription factor. The expression of genes encoding proteins that transform the vegetative cell into a heterocyst is then activated with a spatiotemporal specificity to produce a mature functional heterocyst. Recent global analyses have added information on the



kinetics and levels of gene expression during the process of differentiation, and much information is also available concerning the complexity of the promoter regions of a number of these genes. Understanding the molecular mechanism of operation of these promoters, including the roles of HetR and NtcA, is a major goal of research in this field.

We express our gratitude to the contributing authors for their dedicated efforts to clearly expose to researchers and students the latest advances in their respective multidisciplinary fields. We hope the readers of this book will share our fascination for cyanobacteria.

**Franck Chauvat and Corinne Cassier-Chauvat**

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## **Environmental and Evolutionary Genomics of Microbial Algae: Power and Challenges of Metagenomics**

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# Structural, Mechanistic and Genomic Insights into OCP-Mediated Photoprotection

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## Abstract

Until relatively recently, photoprotective mechanisms in cyanobacteria were poorly understood. Yet, they play a crucial role in the ecophysiology of cyanobacteria, which inhabit a range of environments, some of them are extreme where stresses such as high salinity, drought and temperature exacerbate the threat of photodamage. This review focuses on the OCP-mediated photoprotective mechanism, which is widespread among cyanobacteria. Mechanistic and structural studies combined with genomic sequence data are painting an increasingly detailed picture of OCP-mediated photoprotection.



## 1. INTRODUCTION

The conversion of solar into chemical energy by plants and cyanobacteria is essential to life on earth. However, when the amount of light energy exceeds the capacity of these organisms' photosynthetic apparatus to harness it, the light poses a threat to life. Cyanobacteria, like plants, carry out oxygenic photosynthesis using two macromolecular assemblies, Photosystem I (PSI) and Photosystem II (PSII), linked by an electron transport chain. In conditions of excessive light, the photosynthetic electron transport chain becomes stalled in a reduced state and reactive oxygen species (ROS) are formed which leads to severe cell damage. Nutrient starvation and low CO<sub>2</sub> conditions predispose photosynthetic organisms to this threat at even relatively low irradiance.

Cyanobacteria have evolved at least two key photoprotective mechanisms to cope with abrupt and fluctuating changes in the quality and intensity of light: State transitions (reviews, e.g. [Minagawa, 2010](#); [Rochaix, 2010](#); [Wollman, 2001a](#)) and the orange carotenoid protein (OCP)-related nonphotochemical quenching (NPQ) (previous reviews: [Bailey & Grossman, 2008](#); [Karapetyan, 2007](#); [Kerfeld, Alexandre, et al., 2009](#); [Kerfeld & Kirilovsky, 2010](#); [Kirilovsky, 2007](#); [Kirilovsky & Kerfeld, 2012](#)). Both involve rapid changes (seconds to minutes) in the photosynthetic apparatus and a decrease in the effective size of the PSII antenna, but by very different means.

To understand these photoprotective mechanisms, it is necessary to place them in the context of the distinctive light-harvesting antenna of cyanobacteria. In cyanobacteria, the major light-harvesting antenna is an extramembranous complex known as the phycobilisome. Phycobilisomes are composed of several types of chromophorylated phycobiliproteins and linker peptides needed for structural organization and function (for reviews, see [Adir, 2005](#); [Glazer, 1984](#); [Grossman, Schaefer, et al., 1993](#); [MacColl, 1998](#); [Tandeau de Marsac, 2003](#)). Phycobilisomes have a trimeric core from which rods radiate. The major core protein is allophycocyanin (APC). The rods are more variable: in most freshwater cyanobacterial species, the rods contain only phycocyanin (PC), whereas in many marine cyanobacteria, phycoerythrin (PE) or phycoerythrocyanin (PEC) are found in the distal end of the rods. These complexes are attached to the outer surface of the thylakoid membranes ([Gantt & Conti, 1966](#)) via the large, chromophorylated, core membrane linker protein L<sub>CM</sub> (ApcE) ([Redlinger & Gantt, 1982](#)), which

also serves as the terminal energy emitter. Two other chromophorylated proteins, ApcD and ApcF, also function as terminal emitters. Harvested light energy is transferred from the terminal emitters to the chlorophylls of PSII and PSI (Mullineaux, 1992; Rakhimberdieva, Boichenko, et al., 2001).

One type of cyanobacterial photoprotective mechanism, known as State Transitions is triggered by light at the level of the phycobilisome and involves regulation of the energy distribution between the two photosystems under low light conditions; this is triggered by changes in the redox state of the plastoquinone (PQ) pool (reviews: van Thor, Mullineaux, et al., 1998; Williams & Allen, 1987; Wollman, 2001a). Exposure of cyanobacteria to orange or green light, absorbed predominantly by phycobilisomes, causes a reduction of the PQ pool and a relative decrease of the PSII fluorescence yield is observed (State 2). Conversely, illumination with blue or far red light, preferentially absorbed by PSI, induces the oxidation of the PQ pool and a relative increase of the fluorescence yield is induced (State 1). Two theories have been proposed to explain the State Transition mechanism (for reviews, see Biggins & Bruce, 1989; Mullineaux, 1999; van Thor, Mullineaux, et al., 1998). The first proposes that the phycobilisomes are mobile elements which, by changing their association with PSII and PSI, deliver energy preferentially to one or the other photosystem (Allen, Sanders, et al., 1985; Joshua & Mullineaux, 2004; Mullineaux, Tobin, et al., 1997; Sarcina, Tobin, et al., 2001). The second theory proposes that the mobile elements are the photosystems changing the 'spillover' of energy from PSII to PSI chl *a* molecules (Bruce & Biggins, 1985; Ley & Butler, 1980; Olive, Mbina, et al., 1986). More recently, McConnell et al. (2002) suggested that changes in the redox state of the PQ pool induce independent changes in the energy transfer between the phycobilisomes and the photosystems, and between the photosystems themselves. Nothing is known about how the reduction or oxidation of the PQ pool could induce the movement of phycobilisomes or photosystems.

In contrast, the OCP-related NPQ mechanism is induced by strong blue or white light intensities (El Bissati, Delphin, et al., 2000; Rakhimberdieva, Stadnichuk, et al., 2004; Wilson, Ajlani, et al., 2006). Light activates a soluble carotenoid protein, the Orange Carotenoid Protein (OCP), which interacts with the phycobilisomes to increase thermal dissipation of absorbed energy, resulting in a decrease of energy arriving at the reaction centres (Wilson, Ajlani, et al., 2006; Wilson, Punginelli, et al., 2008). The increase of thermal dissipation also causes a decrease of the yield of phycobilisome fluorescence creating a nonphotochemical fluorescence quenching. This review focuses

on the emerging understanding of the OCP-mediated photoprotective mechanism, and its structural basis within the context of new genomic information.



## **2. THE OCP-BASED NPQ MECHANISM IS PHOTOPROTECTIVE**

### **2.1. Initial Observations**

The first result that suggested the existence of a photoprotective mechanism involving energy and fluorescence quenching at the level of the phycobilisome was published in 2000 (El Bissati, Delphin, et al., 2000). The authors showed that strong blue light induced a large fluorescence quenching that was not related to photoinhibition or State Transitions. Several subsequent publications proposed that this blue-light-induced quenching, which also increased in iron starvation conditions, was related to the IsiA protein (Bailey, Mann, et al., 2005; Cadoret, Demouliere, et al., 2004; Joshua, Bailey, et al., 2005), a member of the light harvesting protein family. Under some conditions, IsiA was known to accumulate as large empty rings (without PSI) that are in a strongly quenched state, suggesting that they can be responsible of thermal dissipation of absorbed energy (Ihalainen, D'Haene, et al., 2005; Yermenko, Kouril, et al., 2004). The demonstration that the blue-light-induced fluorescence quenching was related to the phycobilisomes and the OCP (and not to IsiA) came several years later based on the results of three different groups working with IsiA, PSI, PSII and a phycobilisome mutants of *Synechocystis* PCC 6803 (hereafter *Synechocystis*) (Rakhimberdieva, Stadnichuk, et al., 2004; Scott, McCollum, et al., 2006; Wilson, Ajlani, et al., 2006; Wilson, Boulay, et al., 2007). The fluorescence quenching was induced under strong blue light in strains lacking IsiA or PSII but it was absent in strains lacking phycobilisomes or containing only the phycobilisome rods (Rakhimberdieva, Stadnichuk, et al., 2004; Scott, McCollum, et al., 2006; Wilson, Ajlani, et al., 2006; Wilson, Boulay, et al., 2007). The phycobilisome fluorescence quenching did not depend on the redox state of the plastoquinone pool or the *trans*-thylakoid  $\Delta$ pH (El Bissati, Delphin, et al., 2000; Wilson, Ajlani, et al., 2006). In contrast, the action spectrum of phycobilisome fluorescence quenching suggested that a carotenoid could be involved in this mechanism (Rakhimberdieva, Stadnichuk, et al., 2004). Subsequently, Wilson et al. in 2006 demonstrated that a soluble carotenoid protein binding a keto-carotenoid, the OCP, is essential for this mechanism, now known as the OCP-related NPQ mechanism. For a detailed

description of the experiments dealing with the discovery of this mechanism and its first characterization, see [Karapetyan \(2007\)](#), [Kirilovsky \(2007\)](#) and [Bailey and Grossman \(2008\)](#). For more recent reviews, see [Kirilovsky \(2010\)](#) and [Kirilovsky and Kerfeld \(2012\)](#).

## 2.2. The OCP Induces a Decrease of the Effective Antenna Size

The OCP-mediated fluorescence quenching correlates with a decrease of the effective size of the antenna and in the amount of energy arriving at the reaction centres ([Gorbunov, Kuzminov, et al., 2011](#); [Rakhimberdieva, Elanskaya, et al., 2010](#); [Wilson, Ajlani, et al., 2006](#)). Measurements of oxygen evolving activity at different light intensities showed that PSII activity saturates at higher light intensities in ‘quenched’ cells than in ‘non-quenched’ cells ([Wilson, Ajlani, et al., 2006](#)). Using *Synechocystis* mutants lacking the PSII or PSI and measuring PSI (or PSII) activities, [Rakhimberdieva, Elanskaya, et al. \(2010\)](#) demonstrated that only 60–70 % of the energy absorbed by the phycobilisome arrives at the reaction centres. In *Synechocystis* and *Synechococcus* sp. CCMP 1379 cells grown at high light intensities (600  $\mu\text{mol photons}$ ), strong blue light can induce about 63% of fluorescence quenching that was correlated to a decrease of the functional cross-section of PSII by 53% ([Gorbunov, Kuzminov, et al., 2011](#)). Under low-light growth conditions, the magnitude of fluorescence quenching was minimal ([Gorbunov, Kuzminov, et al., 2011](#)). The amplitude of fluorescence quenching depends on the concentration of the OCP. In wild-type (WT) *Synechocystis* cells (grown at 60  $\mu\text{mol photons}$ ), which contain one OCP per 2–3 phycobilisomes, a maximum fluorescence quenching of 35% is observed, whereas in a mutant strain containing about 8 times more OCP (3–4 OCP per phycobilisome), 65–70% of fluorescence quenching is observed ([Kirilovsky & Kerfeld, 2012](#); [Wilson, Punginelli, et al., 2008](#)). The expression of the OCP also increases under different stress conditions (high light ([Hihara, Kamei, et al., 2001](#)), salt stress ([Fulda, Mikkat, et al., 2006](#)), iron starvation ([Wilson, Boulay, et al., 2007](#))) and oxidative stress ([Blot, Daniella Mella-Flores, et al., 2011](#)). Under iron starvation and high-light growth conditions, a larger fluorescence quenching correlating with a greater OCP concentration was observed ([Boulay, Abasova, et al., 2008](#); [Gorbunov, Kuzminov, et al., 2011](#); [Wilson, Boulay, et al., 2007](#)). Thus, since the maximum amplitude of fluorescence quenching and the extent of the decrease of the effective functional antenna size depends on the amount of the OCP in the cell, different amplitudes of fluorescence quenching could be observed under different growth conditions. These results suggested that the OCP-related photoprotection



becomes more important in the presence of combined stress, especially under stress conditions that induced decrease of PSI complexes and an imbalance between the number of phycobilisomes and reaction centres.

The OCP-induced thermal dissipation of absorbed energy protects the cells from photodamage. A  $\Delta$ OCP *Synechocystis* mutant is more sensitive to high light intensities than WT; exposure of mutant cells to strong irradiance caused a more rapid decrease of PSII activity than in WT cells (Wilson, Ajlani, et al., 2006). Moreover, cyanobacterial strains lacking the OCP, such as *Thermosynechococcus elongatus* and *Synechococcus elongatus*, lost their PSII activity faster under fluctuating high light conditions than strains containing the OCP (Boulay, Abasova, et al., 2008).

Thus, cyanobacteria protect themselves from high irradiance by decreasing the effective antenna size of PSII. The antenna size must be fully restored when cells are again under low light conditions to maintain optimum growth. Another protein, the fluorescence recovery protein (FRP; discussed below), plays a critical role in this process (Boulay, Wilson, et al., 2010). When ‘quenched’ cyanobacteria cells are to low light conditions or are incubated in darkness, a recovery of the lost phycobilisome fluorescence is observed (Boulay, Wilson, et al., 2010; Rakhimberdieva, Bolychevtseva, et al., 2007b; Wilson, Ajlani, et al., 2006). The fluorescence recovery is strongly temperature dependent (Gorbunov, Kuzminov, et al., 2011; Rakhimberdieva, Bolychevtseva, et al., 2007b; Wilson, Punginelli, et al., 2008). Interruption of the FRP gene in *Synechocystis* largely inhibits fluorescence recovery (Boulay, Wilson, et al., 2010).

### 3. OCP-MEDIATED PHOTOPROTECTION FROM A STRUCTURAL PERSPECTIVE

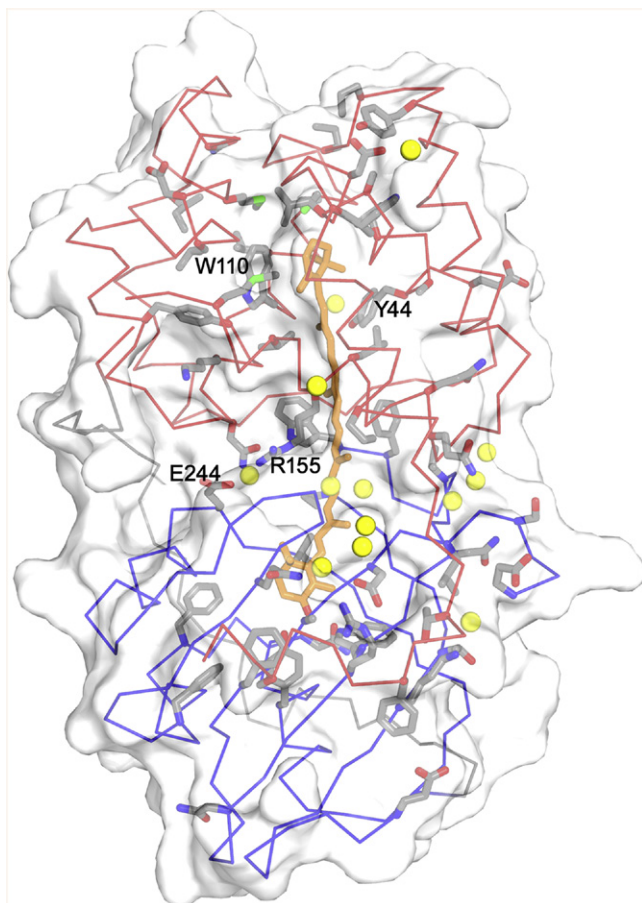
#### 3.1. Crystal Structures of the OCP

David Krogmann et al. first isolated the OCP in the course of purification of c-type cytochromes. They described it as a soluble protein containing the keto-carotenoid 3-hydroxyequinenone (hECN) (Holt & Krogmann, 1981) and demonstrated that it was present in three cyanobacterial strains: *Arthrospira maxima*, *Microcystis aeruginosa* and *Aphanizomenon flos-aqua*. Later, Krogmann’s group isolated the OCP from *A. maxima* and *Synechocystis* (Wu & Krogmann, 1997). Using protein sequencing methods to obtain the primary structure of the N-terminus of the OCP, they were able to deduce that it was encoded by the *slr1963* gene in the then newly sequenced genome of *Synechocystis* PCC 6803 (Wu & Krogmann, 1997).

In 2003, before any functional studies had been initiated on the OCP, Kerfeld, Sawaya, et al. (2003) reported the crystal structure of the *A. maxima* OCP isolated by Krogmann et al. More recently, Kerfeld's group has structurally characterized the *Synechocystis* OCP (Wilson, Kinney, et al. 2010). The OCP structures (Fig. 1.1), presumably both in the resting form, are essentially identical, composed of two domains: an  $\alpha$ -helical N-terminal domain (residues ~19–165) that is unique to cyanobacteria (Pfam 09150) and an  $\alpha/\beta$  C-terminal domain (residues ~190–317) that is a member of the widely distributed nuclear transport factor 2 (NTF2) fold superfamily (Pfam 02136). The two domains are joined by a long (~25 amino acids) flexible linker. The multiple structures now available also suggest that there are structurally conserved water molecules between domains and surrounding the carotenoid. The functional significance of these is unknown, but they could facilitate protein conformational changes. The carotenoid, 3-hECN, spans both domains of the protein (Kerfeld, Sawaya, et al., 2003; Wilson, Kinney, et al., 2010). hECN is a keto-carotenoid with a conjugated chain of 11 carbon-carbon double bonds; in the OCP structures, these are in all-trans configuration (Kerfeld, Sawaya, et al., 2003; Polivka, Kerfeld, et al., 2005; Wilson, Punginelli, et al., 2008). The hECN keto- (carbonyl) group is hydrogen bonded to absolutely conserved (Fig. 1.2) Tyr 201 and Trp 288 residues within an otherwise hydrophobic pocket of the C-terminal domain (Kerfeld, Sawaya, et al., 2003; Wilson, Kinney, et al., 2010; Wilson, Punginelli, et al., 2011). The hydroxyl ring of hECN in the N-terminal domain is nestled within a group of conserved aromatic residues (Trp 41, Tyr 44 and Trp 110) (Kerfeld, Sawaya, et al., 2003; Wilson, Kinney, et al., 2010; Wilson, Punginelli, et al., 2011).

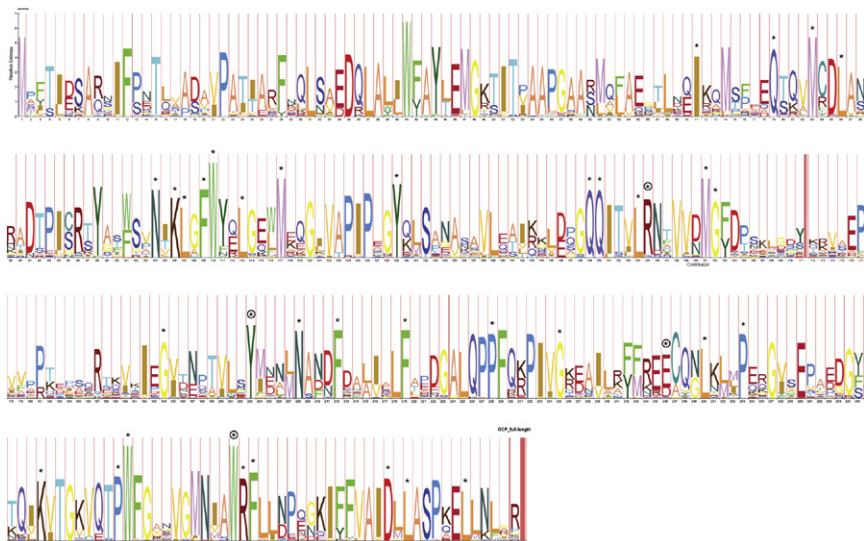
### 3.2. Clues to the Structural Basis of Photoactivity

In the crystal structures of the OCP, the carotenoid is only sparingly surface exposed, suggesting that some degree of structural rearrangement would be necessary for it to be competent for energy transfer from the phycobilisome. This hypothesis is supported by light-minus-dark differential FTIR spectra showing that light absorption by OCP induces conformational changes in the protein. These changes were interpreted to correspond to a less-rigid helical structure (loosening of  $\alpha$ -helices) and a compaction (strengthening) of the  $\beta$ -sheet domain with additional loop changes (Wilson, Gwizdala, et al., 2012; Wilson, Punginelli, et al., 2008). These changes are essential for OCP interaction with the phycobilisome (Gwizdala, Wilson, et al., 2011; Wilson, Gwizdala, et al., 2012).



**Figure 1.1** The structure of the OCP. The N-terminal domain is uppermost, with the backbone traced in red; the C-terminal domain is traced in red. The carotenoid is shown in sticks as are all absolutely conserved amino acids among the 90 currently available OCP sequences. Conserved water molecules are shown as spheres. Figure made with pymol <http://www.pymol.org/>. See the colour plate.

In 2008, the OCP was shown to be a photoactive protein (Wilson, Punginelli, et al., 2008). In darkness, it is orange (OCP<sup>o</sup>) and its absorbance spectrum shows two maxima at 476 and 496 nm, with a shoulder at 440 nm. Absorption of blue-green light converts, with a very low yield, the OCP<sup>o</sup> form to a metastable red form (OCP<sup>r</sup>) (Wilson, Punginelli, et al., 2008). The red-shifted spectrum of the OCP<sup>o</sup> loses the resolution of the vibrational bands and shows a large maximum at 510 nm. The rates of



**Figure 1.2** Hidden Markov model logo of the primary structure of the OCP. Absolutely conserved residues are denoted with stars. Sequence numbering corresponds to that of *Synechocystis* OCP. The HMM logo was derived from the multiple sequence alignment of the primary structures of the OCP. The HMM was built using HMMER (Eddy 1998), and the logo was visualized using LogoMat-M. (Schuster-Boeckler, J. et al. 2004). For colour version of this figure, the reader is referred to the online version of this book.

OCP<sup>r</sup> accumulation largely depend on light intensity (Wilson, Punginelli, et al., 2008) and, in a less-pronounced manner, on temperature (Wilson, Gwizdala, et al., 2012). *In vitro*, in darkness, OCP<sup>r</sup> spontaneously converts to OCP<sup>o</sup>. This reaction shows strong temperature dependence (Wilson, Punginelli, et al., 2008).

The OCP<sup>r</sup> is the active form of the protein that mediates energy and fluorescence quenching at the level of phycobilisomes (Gwizdala, Wilson, et al., 2011; Punginelli, Wilson, et al., 2009; Wilson, Kinney, et al., 2010; Wilson, Punginelli, et al., 2008). The presence of the hydrogen bonding between the carotenoid carbonyl group and the C-terminal domain of the OCP is essential for photoactivity. In the absence of hECN and echinenone (ECN), OCP binds zeaxanthin (lacking a keto group) but the resulting yellow zeaxanthin–OCP protein is not photoactivate and it is unable to induce fluorescence quenching (Punginelli, Wilson, et al., 2009). Also, the replacement of either Tyr 201 or Trp 288 by other amino acids renders the OCP photoinactive and unable to induce photoprotection (Wilson, Punginelli, et al., 2011). In contrast, the hydroxyl group does

not play a role in photoactivation. When hECN and zeaxanthin are absent, ECN (lacking the hydroxyl group) binds to the apo-protein and the ECN–OCP is both photoactive and able to induce the photoprotective mechanism (Punginelli, Wilson, et al., 2009; Wilson, Punginelli, et al., 2011). No differences in the rates of photoactivation or in the stability of the red forms of hECN–OCP and ECN–OCP were detected. While the hydroxyl group is not needed for photoactivation, the interaction of the hECN hydroxyl ring with aromatic amino acids is known to be essential for the formation and/or stabilization of OCP<sup>r</sup>. When Tyr 44 or Trp 110 are replaced by nonaromatic amino acids, the OCP remains orange under illumination and does not induce photoprotection (Wilson, Punginelli, et al., 2008; Wilson, Punginelli, et al., 2011).

Light absorption induces changes in the position and conformation of the keto-carotenoid; its apparent conjugation length increases by about one conjugated bond and adopts a less distorted, more planar structure (Wilson, Punginelli, et al., 2008). Modifications of the excited states of the carotenoid are associated with these conformational changes (Berera, van Stokkum, et al., 2012). Binding of hECN to the protein is responsible for shortening of the S1 lifetime (from 6.5 ps in solution to 3.3 ps) in the OCP and for the stabilization of an intramolecular charge-transfer (ICT) state, making the hECN–OCP a more effective energy dissipator than free carotenoid (Berera, van Stokkum, et al., 2012; Polivka, Kerfeld, et al., 2005). It was also shown that the hydrogen bonds between the carbonyl group of the carotenoid and the C-terminal domain of the OCP play a crucial role in modulating and stabilizing the ICT state (Berera, van Stokkum, et al., 2012; Chábera, Durchan, et al., 2010; Polivka, Kerfeld, et al., 2005). In the excited state of OCP<sup>r</sup>, the contribution of the ICT state is stronger than in OCP<sup>o</sup> (Berera, van Stokkum, et al., 2012), increasing the potential for the carotenoid to be a mediator in the energy dissipation process. The efficiency of energy quenching increases with the strength of the ICT state (Berera, Herrero, et al., 2006). It was proposed that OCP<sup>r</sup> accepts energy from an excited bilin of the phycobilisome via its ICT state. The ICT state partially decays to the ground state and partially to the S1 state, which in turn also decays to the ground state (Berera, van Stokkum, et al., 2012). The lifetimes of the ICT and S1 states (0.6 and 3.2 ps), which are about three orders of magnitude shorter than that of the excited bilin, make the hECN a very efficient quencher. Thus, energy absorbed by the phycobilisomes can be harmlessly dissipated as heat.

### 3.3. Structural Similarities to Other Blue-Light-Responsive Proteins

In addition to allowing the dissection of the roles of specific amino acids in photoactivity and photoprotective function, the three-dimensional structure of the OCP provides a basis for comparison to other well-characterized photoactive proteins. The ability to respond to blue light is not restricted to photosynthetic organisms, it is widespread in nature. In the last decade, in addition to the emergence of understanding of the OCP response to blue light, there have been tremendous advances in understanding the structural basis of function in proteins that contain blue-light-responsive BLUF (Blue light using flavin) and LOV (light oxygen voltage) domains (reviewed in [Losi, 2007](#)). BLUF and LOV domains typically function as receiver/input domains that can be either covalently or noncovalently associated with various output domains for diverse functions such as signal transduction, enzymatic activity or DNA binding ([Losi, 2007](#); [Nash, McNulty, et al., 2011](#)). There is a superficial structural similarity between BLUF and LOV domains and the C-terminal domain of the OCP: all contain a core beta sheet surrounded by helices. In BLUF and LOV domains, changes in the chromophore in response to light cause modifications of hydrogen bonding patterns among the helices and the beta sheet and changes in exposure of discrete parts of the structure to facilitate specific protein–protein interactions in response to the light signal. For example, in some LOV-based photoresponsive systems, light alters the hydrogen bonding to the chromophore, which is propagated through the beta sheet, culminating in the displacement of a helix ( $J\alpha$ ) that otherwise interacts effector domain ([Harper, Christie, et al., 2004](#); [Harper, Neil, et al., 2003](#)). Likewise, in many of these systems, structurally conserved water molecules are also known to play a role in the conformational changes that convert the light signal into an effector function.



## 4. THE OCP–PHYCOBILISOME INTERACTION

### 4.1. The *In Vitro* Reconstitution System: Only OCP<sup>r</sup> Interacts With Phycobilisomes

The recent development of an *in vitro* reconstitution system for the OCP-related photoprotective mechanism provides new approaches to the characterization of the OCP–phycobilisome interaction ([Gwizdala, Wilson, et al., 2011](#)). When phycobilisomes are illuminated with strong

white or blue-green light in the presence of an excess of the OCP, a large phycobilisome fluorescence quenching is observed (Gwizdala, Wilson, et al., 2011; Stadnichuk, Yanyushin, et al., 2011). An excess of the OCP in the experimental system is necessary because a high concentration of phosphate (0.8 M or higher) is needed to maintain the integrity of purified phycobilisomes (Gwizdala, Wilson, et al., 2011; Wilson, Gwizdala, et al., 2012). By re-isolating phycobilisomes after incubation in light or in darkness with WT-OCP or Tyr44-OCP (nonphotoactive), it was demonstrated that only OCP<sup>r</sup> is able to bind to phycobilisomes (Gwizdala, Wilson, et al., 2011). In addition, it was also shown that only one OCP<sup>r</sup> per phycobilisome is sufficient to quench almost all of the phycobilisome fluorescence (Gwizdala, Wilson, et al., 2011).

When phycobilisomes are illuminated in the presence of OCP<sup>o</sup>, the rate and amplitude of quenching depends on light intensity because the accumulation of OCP<sup>r</sup> depends on light intensity. In contrast, fluorescence quenching does not depend on light intensity and occurs even in darkness when phycobilisomes are incubated with OCP<sup>r</sup>, indicating that OCP<sup>r</sup> binding to phycobilisomes is light independent (Gwizdala, Wilson, et al., 2011). This was also observed *in vivo*: in whole *Synechocystis* cells, when fluorescence quenching is induced by a brief period of intense light, fluorescence quenching continues to occur in darkness for several minutes (Gorbinov, Kuzminov, et al., 2011; Rakhimberdieva, Kuzminov, et al., 2011).

The OCP<sup>r</sup> is also able to quench all the fluorescence of a mutant phycobilisome lacking the rods (CK phycobilisomes, containing only the core) but it is unable to quench rod fluorescence when the core is absent (Gwizdala, Wilson, et al., 2011). This confirms the hypothesis that the OCP binds the core of the phycobilisome; this was suggested by *in vivo* experiments with phycobilisome mutants (Scott, McCollum, et al., 2006; Stadnichuk, Lukashev, et al., 2009; Wilson, Ajlani, et al., 2006). The OCP<sup>r</sup>-core complexes are less stable than the OCP<sup>r</sup>-whole phycobilisome complexes, indicating that rods (formed by three PC hexamers) stabilize OCP<sup>r</sup> binding to phycobilisomes (Gwizdala, Wilson, et al., 2011). The presence of only one PC hexamer in CB phycobilisomes (core plus rods containing one PC hexamer) is sufficient to stabilize the binding (Gwizdala, Wilson, et al., 2011).

## 4.2. The Site of Energy and Fluorescence Quenching in the Phycobilisome Core

The core of *Synechocystis* phycobilisomes is composed of three cylinders, formed by four APC trimers. The trimers of the upper cylinder are formed



by three  $\alpha$ APC– $\beta$ APC heterodimers and have a maximum fluorescence emission at 660 nm (APC<sub>660</sub>). Each basal cylinder contains two APC<sub>660</sub> and two APC<sub>680</sub> trimers. In one APC<sub>680</sub> trimer, one  $\alpha$ APC is replaced by ApcD and in the other one, one  $\beta$ APC is replaced by ApcF and one  $\alpha$ APC by the N-terminal domain of ApcE (or Lcm). Each subunit contains a phycocyanobilin chromophore. The APC<sub>680</sub> trimers transfer the energy absorbed by the phycobilisomes to the chlorophyll antenna and reaction centres. Results described in three recent articles strongly suggest that OCP<sup>r</sup> interacts exclusively with one of the APC<sub>660</sub> trimers (Jallet, Gwizdala, et al., 2012; Tian, Gwizdala, et al., 2012; Tian, van Stokkum, et al., 2011). Another publication suggested that, in addition, the OCP can interact with APC<sub>680</sub> trimers (Kuzminov, Karapetyan, et al., 2012), whereas another proposed that the OCP interacts only with ApcE (Lcm) (Stadnichuk, Yanyushin, et al., 2012). Strong blue green light is able to induce fluorescence quenching in *Synechocystis*-mutant cells lacking ApcD or ApcF or both (Jallet, Gwizdala, et al., 2012; Stadnichuk, Yanyushin, et al., 2012). Moreover, *in vitro* reconstitution experiments using phycobilisomes isolated from these mutants and from WT cells showed similar amplitude and kinetics of fluorescence quenching and recovery under different OCP and phosphate concentrations (Jallet, Gwizdala, et al., 2012). Finally, in *Anabaena* PCC7120, the absence of ApcD did not affect the strong blue–green–light-induced fluorescence quenching (Dong, Tang, et al., 2009). Thus, three different research groups have shown that ApcD and ApcF are not required for OCP-related fluorescence quenching.

Because the absence of ApcE leads to the lack of phycobilisomes, the role of the chromophore was probed by mutation; Cys 190, which binds the phycocyanobilin, was changed to a Ser (Jallet, Gwizdala, et al., 2012). The C190S-mutant ApcE binds a chromophore that emits at 710 nm, most probably a (3Z)-phycocyanobilin (Gindt, Zhou, et al., 1994). In isolated phycobilisomes, this chromophore is easily lost during illumination with strong white light (Jallet, Gwizdala, et al., 2012). When the chromophore (emitting at 710 nm)-lacking phycobilisome was illuminated in the presence of the OCP, a large fluorescence quenching was observed. No differences were detected in the fluorescence quenching between the ApcE mutant and WT cells (Jallet, Gwizdala, et al., 2012). Thus, it was concluded that ApcE is also not needed for fluorescence quenching and that, most probably, the OCP binds to an APC<sub>660</sub> trimer. However, Stadnichuk, Yanyushin, et al. (2012) presented results that can suggest that OCP interacts with ApcE. They isolated ApcE and illuminated the protein in the presence of increasing



concentrations of the OCP. They observed a small fluorescence quenching of the ApcE chromophore that increased with OCP concentration. ApcE is very hydrophobic and nearly insoluble in most buffers. It can be kept in solution only in the presence of 2M urea and formic acid at pH 2–3. Under these conditions, ApcE and OCP are both somewhat denatured and this denaturation could induce an interaction between the OCP and the ApcE chromophore that does not exist when ApcE is in the phycobilisome.

Tian et al. (2011, 2012) used spectrally resolved picosecond fluorescence measured by a streak-camera to elucidate the site of quenching in the phycobilisome. They studied *Synechocystis* cells (WT,  $\Delta$ OCP and overexpressing OCP) and isolated phycobilisomes (WT, CK (only the core) and CB (the core plus rods containing only one PC hexamer)) in quenched and unquenched states. A compartmental model was constructed to fit the data and describe excitation energy transfer and trapping using target analysis. All spectra and most of the transfer rates are nearly identical between cells and isolated phycobilisomes (Tian, Gwizdala, et al., 2012; Tian, van Stokkum, et al., 2011). The fitting of the quenched state used an additional decay rate,  $k_q$ , to various compartments and keeping all other rates as for unquenched samples. Only quenching at the  $APC_{660}$  compartment led to a satisfactory fit to the data in cells and isolated phycobilisomes (Tian, Gwizdala, et al., 2012; Tian, van Stokkum, et al., 2011). In cells, the overall quenching rate was  $(16 \pm 4 \text{ ps})^{-1}$  (Tian, van Stokkum, et al., 2011). In isolated WT and CB phycobilisomes, it is (only) somewhat slower  $(33 \pm 3 \text{ ps})^{-1}$  and  $(39 \pm 4 \text{ ps})^{-1}$  respectively (Tian, Gwizdala, et al., 2012). Since only one of the 66  $APC_{660}$  pigments present in the core is directly quenched by the OCP, in cells, the molecular quenching rate of  $APC_{660}^Q$  is at most  $(240 \pm 60 \text{ fs})^{-1}$ , which is extremely fast and leads to efficient quenching (80%). In isolated WT phycobilisomes, the molecular quenching is also very fast,  $(500 \pm 50 \text{ fs})^{-1}$  or faster. Tian et al. proposed that this fast quenching is most probably caused by a charge transfer between  $APC_{660}^Q$  and the hECN of OCP in its activated form; however, they did not discard the possibility that excitation energy transfer (EET) from  $APC_{660}^Q$  to hECN could be responsible for quenching (Tian, Gwizdala, et al., 2012; Tian, van Stokkum, et al., 2011). Berera, van Stokkum, et al. (2012), based on the characteristics of the excited states of OCP<sup>r</sup>, prefer the latter hypothesis. Ultrafast transient absorption measurements of OCP–phycobilisome complexes will be needed to distinguish between these two possibilities.

Kuzminov, Karapetyan, et al. (2012) used nonlinear laser fluorimetry to elucidate the site of quenching in the phycobilisome. They measured

fluorescence emission spectra of *Synechocystis* cells lacking both photo-systems under excitation at 532 nm in a wide range of laser photon flux density. Deconvolution of the fluorescence spectra into three Gaussians assigned to PC, APC<sub>660</sub> and APC<sub>680</sub> fluorescence and analysis of the non-linear dependence of fluorescence and the laser photon flux density allowed them to conclude that both APC<sub>660</sub> and APC<sub>680</sub> are quenched (Kuzminov, Karapetyan, et al., 2012). Thus, the possibility that the OCP binds an APC<sub>680</sub> trimer in some phycobilisomes or that OCP binding allows the simultaneous quenching of 660 and 680 nm emission remains an open question. Nevertheless, based on our knowledge of the stringent specificity of OCP binding and of the interaction between the OCP<sup>r</sup> and the phycobilisome, we think that this is not likely.

### 4.3. What Part of the OCP Interacts with the Phycobilisome?

Several recent experiments have provided the first insights into the structural basis of the interaction between the OCP and phycobilisome to mediate quenching. To dissect out the role of specific amino acids, it is first necessary to consider the structural anatomy of the OCP. As noted above, the OCP is composed of two domains joined by a flexible linker (Fig. 1.1). Given that the OCP-mediated photoprotective mechanism requires the OCP<sup>o</sup> to OCP<sup>r</sup> conversion, and is known from FTIR measurements to involve protein motion, and to be sensitive to viscosity, the assumption is that there are protein conformation changes that are requisite for photoprotection; perhaps these are important for exposing and orienting the carotenoid.

The two domains interact through two regions (Fig. 1.1). The first 19 amino acids of the OCP extend from the N-terminal domain and form part of the C-terminal domain in full-length OCP. This N-terminal inter-domain interface buries 947 and 775 Å<sup>2</sup> of the N- and C-terminal domains, respectively, and is composed of conserved residues from both domains (Wilson, Kinney, et al., 2010). The second interface, across which the carotenoid spans the protein, buries 628 and 723 Å<sup>2</sup> of the N- and C-terminal domains, respectively. It contains a salt bridge between R155 and E244. This salt bridge stabilizes the interaction between the two domains (Fig. 1.1). Interestingly, the OCP mutants R155L and R155E are photoactive but unable to induce fluorescence quenching in *Synechocystis* cells (Wilson, Gwizdala, et al., 2012; Wilson, Kinney, et al., 2010). *In vitro*, accumulation of the red form of R155L-OCP and R155E-OCP is faster than that of WT-OCP and their OCP<sup>r</sup> forms are more stable than the WT-OCP<sup>r</sup> (Wilson, Gwizdala, et al., 2012). The E244L-OCP mutant shows similar

characteristics, implying that the salt bridge between Arg 155 and Glu 244 stabilizes the OCP<sup>o</sup> and strongly suggests that in the red form. This region of interaction between the two domains is weakened or non-existent (Wilson, Gwizdala, et al., 2012). In addition, R155L- and R155E-OCPs are substantially impaired in phycobilisome binding. The change to a neutral amino acid in R155L decreases the strength of OCP<sup>r</sup> binding and the change to a negative charge in R155E OCP almost completely abolishes it. The importance of the positive charge of Arg 155 for binding to the phycobilisome is underscored by the relatively small effect on fluorescence quenching in an R155K mutant (Wilson, Gwizdala, et al., 2012). In contrast, binding of E244L-OCP to phycobilisomes is similar to that of WT-OCP, indicating that this amino acid is not directly involved in the interaction. These results demonstrated that the surface of the N-terminal domain containing the Arg 155 is directly involved in the binding of OCP<sup>r</sup> to the core of phycobilisome. This implies that in the orange form, the presence of the R155–E244 salt bridge stabilizes a ‘closed’ conformation precluding the OCP<sup>o</sup> binding to the phycobilisomes. Accordingly, a model is emerging for the OCP interaction with the phycobilisome: strong blue-green light, by inducing carotenoid (and concomitant protein) conformational changes, causes the breakage of the R155–E244 salt bridge; the resulting domain motion exposes the surface of the N-terminal domain containing Arg155 for interaction with the phycobilisome core, possibly with the negative charges in one of the APC trimers of the phycobilisome core, close to one of the bilin chromophores. It seems that there is only one very specific site of OCP binding and the interaction between Arg155 and APC permits a closer interaction between hECN and one APC chromophore but probably less with two bilins.



## 5. DISCOVERY AND CHARACTERIZATION OF THE FLUORESCENCE RECOVERY PROTEIN

*In vitro*, the isolated OCP<sup>r</sup> spontaneously reverts to OCP<sup>o</sup> in darkness (Wilson, Punginelli, et al., 2008). In *Synechocystis*, however, attachment of OCP<sup>r</sup> to the phycobilisomes stabilizes the OCP<sup>r</sup> and another protein is needed to induce OCP<sup>r</sup> to OCP<sup>o</sup> conversion and detachment from the phycobilisome (Boulay, Wilson, et al., 2010; Gwizdala, Wilson, et al., 2011). In the *Synechocystis* genome, the *slr1963* gene encoding the OCP is followed by a gene (*slr1964*) encoding a conserved protein; the co-occurrence of this gene adjacent to that for the OCP in most available cyanobacterial

genomes has long been noted, but the lack of either sequence homology to any characterized protein and of any experimental data for a functional link to the OCP left the role of this protein enigmatic (Kerfeld, Alexandre, et al., 2009). When this gene was interrupted, only 20–30% of the lost fluorescence during the cell incubation under strong light was recovered (Boulay, Wilson, et al., 2010). The *slr1964* gene product was then named ‘fluorescence recovery protein’ (FRP). The FRP is a soluble 12–13 kDa protein and does not bind a chromophore (Boulay, Wilson, et al., 2010). Co-immunoprecipitation experiments demonstrated that the FRP interacts with OCP<sup>r</sup> (Boulay, Wilson, et al., 2010). *In vitro*, the presence of the FRP greatly accelerated the OCP<sup>r</sup> to OCP<sup>o</sup> conversion (Boulay, Wilson, et al., 2010). Experiments carried out with mutated OCPs suggested that the FRP could act by lowering the energy of activation of the OCP<sup>r</sup> to OCP<sup>o</sup> conversion (Wilson, Gwizdala, et al., 2012). When the FRP is added to OCP<sup>r</sup>–phycobilisome complexes, it induces or accelerates the fluorescence recovery, indicating that it promotes the detachment of OCP from the phycobilisomes (Gwizdala, Wilson, et al., 2011). Crystals of FRP that diffract to 3 Å have been reported (Liu, Shuai, et al., 2011) and the 2.7 Å structure has recently been determined (Kerfeld et al., manuscript in preparation).



## 6. INSIGHTS FROM GENOMIC DISTRIBUTION OF COMPONENTS OF THE OCP PHOTOPROTECTIVE MECHANISM

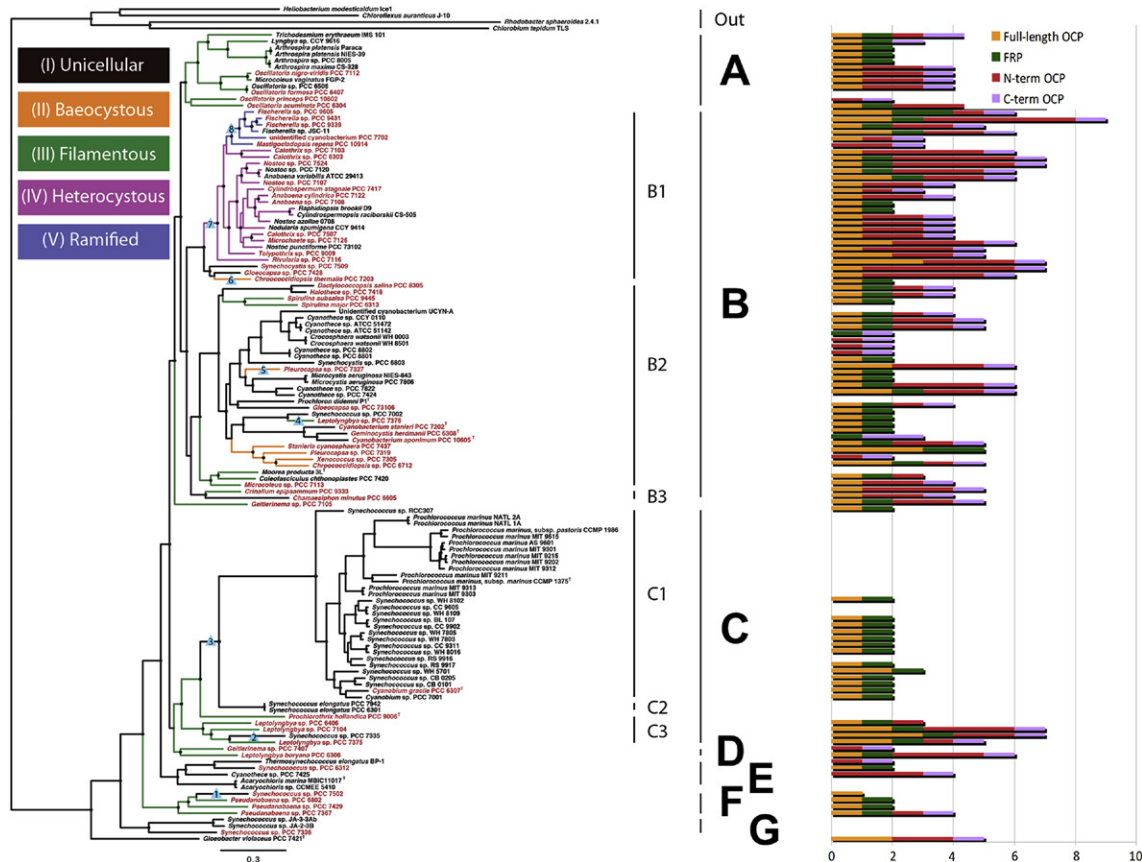
### 6.1. Distribution of Genes Encoding the OCP

With the recent completion of a large-scale cyanobacterial sequencing project (54 phylogenetically diverse cyanobacterial genomes: Shih et al., submitted), comparative genomics of the OCP/FRP system is poised to contribute to our understanding of the OCP-mediated photoprotective mechanism. The potential of genomic information to complement structural and mechanistic studies of OCP function was already apparent at the time of the initial structure determination. Kerfeld et al. noted that homologues to *slr1963* were present only in cyanobacteria containing phycobilisomes. Likewise, they pointed out the presence of shorter OCP paralogues. A survey of genomic sequence data to-date (a total of 129 genomes which includes the 54 new cyanobacterial genomes; Shih et al., submitted) shows that genes encoding the full-length OCP are found in the majority of cyanobacteria (Fig. 1.3); 90 of 127 total genomes surveyed contain at least one gene for a full-length OCP. Most of the organisms that lack the OCP are found in the

marine picocyanobacterial clade. Nineteen of the 90 genomes that contain one full-length OCP contain a second or even a third copy (*Synechococcus* sp. PCC7335, *Synechocystis* sp. PCC7509, *Pleurocapsa* sp. PCC7319).

Numerous cyanobacterial genomes also contain separate genes encoding the N- and C-terminal domains (Fig. 1.3); in some cases, they are adjacent to one another on the chromosome (e.g. in *T. elongatus* and the *Fischerella* genomes); in others, they are found in different locations in the genome (e.g. *Oscillatoria acuminata* PCC6304). Genes for the individual domains can be found in organisms in addition to full-length OCP or as the only homologues to the OCP. In general, most organisms are enriched in additional copies of the N-terminal domain with some diversity in length and sequence. Most of the genomes encoding genes for the N-terminal domain also contain a gene encoding the C-terminal domain, but in contrast to the N-terminal domain paralogues, these are strongly conserved and present only in single copies (Fig. 1.3). However, some organisms contain additional genes encoding proteins that adopt the NTF2 fold that can be detected only by structural similarity (e.g. *ava\_2261* from *Anabaena variabilis*, PDB code: 3dmc and *npun\_r3134* from *Nostoc punctiforme*, PDB code: 2rqj), yet do not have appreciable sequence homology to the C-terminal domain of the OCP. The function of any of these additional copies of the N- and C-terminal domains is unknown; it has been proposed that they could assemble with carotenoids in a modular manner (Kerfeld, Sawaya, et al., 2003) to form OCP variants with different light sensitivities (receivers) or outputs (affinity for phycobilisome or other response). Such modularity, as noted above, is well known for other blue-light-responsive proteins such as those containing BLUF and LOV domains (Losi, 2007). However, *T. elongatus*, in which the N- and C-terminal domains are encoded by two adjacent genes, does not appear to have a characteristic OCP-related photoprotective mechanism (Boulay, Abasova, et al., 2008), suggesting that at least some of these modular paralogues have a different function. It is known that some of the N-terminal domain paralogues are expressed (Anderson, Campbell, et al., 2006; Stockel, Welsh, et al., 2008).

In addition to discovering the OCP, David Krogmann's team was also able to isolate a red protein, dubbed the RCP from *A. maxima* (Knutson, 1998). Sequence analysis and mass spectrometry indicated that this was a short form of the OCP, lacking the first 15 and the last ~150 amino acids of the full-length protein. It was assumed to be a proteolytic fragment of the OCP because the amino acid sequence determined by Edman degradation was otherwise identical to the OCP. Structurally, this corresponds



**Figure 1.3** Current census of the distribution of genes encoding the OCP, FRP and genes for the N- and C-terminal domains of the OCP, based on sequence data described in Shih et al., (in press). Clades and subclades assigned are marked with letters. See the colour plate.

to the loss of the N-terminal helix (that is associated with the C-terminal domain in the intact structure) and the entire C-terminal domain. Now with the availability of genomic sequence data, this can be confirmed to be a proteolytic fragment of the OCP because the *Arthrospira* genome does not contain genes for N-terminal paralogues. Whether proteolytic fragments of the OCP have a functional role (for example, in mediating photoprotection or in proteolysis-based signalling; Turk, Turk, et al., 2012), or are merely degradation products is an open question. Likewise, how the protein and the carotenoid interact in the proteolytic RCP is not at all understood, it is likely that a structural rearrangement occurs to shield the carotenoid from solvent.

A gene for a second type of OCP fragment, missing the first 50 amino acids, had been identified in the genome of *Gloeobacter violaceus*, in addition to one for a full-length OCP. Recently, both forms were shown to be expressed and the fluorescence quenching was attributed to the smaller form of the OCP (Bernát, Schreiber, et al.). However, careful analysis of the *G. violaceus* genome in the region of the glr3935 indicates that there is a more plausible start codon for the gene 150 bp upstream of the one proposed in the databases, suggesting that the gene product of glr3935 is a full-length OCP of 324 amino acids. Both a full length (34 kDa) and a more abundant, lower molecular weight OCP (~30 kDa) were detected in conditions that induced fluorescence quenching; the latter was may also be an indication of proteolytic processing of the OCP.

## 6.2. Distribution of Genes Encoding the FRP

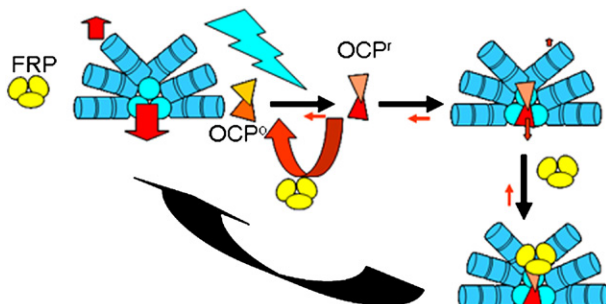
Interestingly, genes for the FRP are not found in all OCP-containing cyanobacteria (see, for example, many of the organisms in clade B1 of Fig. 1.3). Organisms that lack FRP tend to be those enriched in isolated copies of the N-terminal domain (e.g. subclade B1, Fig. 1.3). Organisms with 2–3 copies of the full-length OCP may or may not have a second FRP gene and no organism contains three copies of FRP genes. Homologues to the FRP are almost exclusively found in cyanobacteria, however, credible candidates (similar sized proteins with 35–40% identity) are found in a few bacterial genomes such as that of *Mesorhizobium ciceri* bv *biserrulae* WSM1271. This gene (Mesci\_2682) is part of a gene cluster encoding several transporter homologues.



## 7. A MODEL FOR THE OCP-MEDIATED PHOTOPROTECTIVE MECHANISM

Our current understanding of the OCP-mediated photoprotection is outlined in Fig. 1.4. Blue-green light triggers the activation of the OCP inducing conformational changes in the carotenoid and the protein that





**Figure 1.4** Schematic of the current understanding of the OCP photoprotective mechanism. Light (thunderbolt) activates the OCP (fused triangles) converting OCP<sup>0</sup> into OCP<sup>r</sup>. Only OCP<sup>r</sup> is able to bind to the core of phycobilisomes or to FRP (ovals, here shown as a trimer). These interactions are light independent. Fluorescence quenching depends on the concentration of OCP<sup>r</sup> and on the FRP/OCP ratio. Vertical arrows symbolize energy flow, either to the reaction centre or dissipated as heat. See the colour plate.

are needed for attachment to the phycobilisome and fluorescence quenching (Gwizdala, Wilson, et al., 2011; Wilson, Gwizdala, et al., 2012; Wilson, Punginelli, et al., 2008). Only the activated red protein is able to bind to the phycobilisomes (Gwizdala, Wilson, et al., 2011; Wilson, Gwizdala, et al., 2012). In the cells, the amplitude of fluorescence quenching depends on the concentration of the OCP<sup>r</sup> and on the affinity of OCP<sup>r</sup> for the phycobilisome. The OCP<sup>r</sup> can also interact with FRP, which facilitates rapid reversion to the orange, inactive form. Thus, fluorescence quenching is affected by the presence and concentration of the FRP and on its affinity of OCP<sup>r</sup>. How organisms that lack an FRP accomplish this step is an interesting question although it is known that the OCP<sup>r</sup> can thermally convert back to OCP<sup>0</sup> without interaction with any protein. Thus, the stability of the unbound OCP<sup>r</sup> also influences the rate and amplitude of fluorescence quenching (Gwizdala, Wilson, et al., 2011; Kuzminov, Karapetyan, et al., 2012; Wilson, Gwizdala, et al., 2012). The attachment of OCP<sup>r</sup> to the phycobilisomes stabilizes the red activated form, presumably remaining attached until interaction with the FRP. The phycobilisome-bound OCP<sup>r</sup> quenches the absorbed light energy and fluorescence via charge transfer between a bilin of an APC<sub>660</sub> trimer and the carotenoid of OCP<sup>r</sup> or by EET between the excited state of the bilin and the S1 state of the hECN in OCP<sup>r</sup>. FRP, by interacting with the attached OCP<sup>r</sup>, may induce its conversion to OCP<sup>0</sup> and its almost simultaneous detachment from the phycobilisome. Under strong illumination, a new OCP<sup>r</sup> will rapidly attach to the phycobilisome that will ‘remain’ quenched. In darkness or low light, the concentration of OCP<sup>r</sup> is null or



very low and a recovery of fluorescence and of the size of the antenna will be observed.



## 8. OUTSTANDING QUESTIONS AND FUTURE PROSPECTS

The OCP was first described more than 30 years ago, but in less than a decade after the structure determination of the OCP, a protein with an unknown function, an entire field of OCP-related research has been established. New data from structural, genomic and functional studies are ever more rapidly expanding our understanding of the OCP-mediated photoprotection in cyanobacteria. Key questions that remain to be elucidated include the following:

What are the specific amino acids of the OCP that interact with the phycobilisome?

Does the OCP consist of a receiver and output domain like other blue-light photoreceptors?

What is the structure of the OCP<sup>tr</sup>?

What is the structure of the RCP?

What is the function of the paralogues of the N- and C-terminal domains of the OCP?

How do organisms that lack an FRP recover from NPQ?

How does FRP interact with the OCP and with the phycobilisome?

We anticipate that with the continued rapid accumulation of structural, function and genomic data, these questions will be answered very soon, setting the stage for the tuning of photoprotection in biotechnologically important cyanobacterial systems as well as for developing OCP system for optogenetic applications in synthetic biology.

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# Genomics of Salt Acclimation: Synthesis of Compatible Solutes among Cyanobacteria

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## Abstract

During their long evolution, cyanobacteria were able to inhabit all light-exposed ecosystems. One of the main environmental factors determining cyanobacterial distribution is the salinity of the surrounding medium. Among cyanobacterial strains, three main salt-tolerance groups can be distinguished: low- and moderate-halotolerant cyanobacteria as well as hypersaline strains. Regardless of the final salt resistance, all cyanobacteria apply two basic strategies for a successful acclimation to enhanced salt concentrations: accumulation of compatible solutes combined with active export of toxic ions, particularly  $\text{Na}^+$  and  $\text{Cl}^-$ . During the years 1991–2010, the molecular basis of these mechanisms has been elucidated. Today, many complete genome sequences appear in databases of cyanobacterial strains, which are often difficult to cultivate in the laboratory. These data were used here to screen the genomes of more than 60 cyanobacteria regarding their compatible solute accumulation capacities. Hence, the existing knowledge about cyanobacterial salt acclimation was used to annotate basic salt-resistance mechanisms on the basis of genome information. Understanding the basic salt acclimation among cyanobacteria will also be useful for their future biotechnological application, which will be performed preferentially in saline waters.



## 1. INTRODUCTION

Cyanobacteria can be found in every light-exposed habitat on Earth. The majority of cyanobacterial strains in culture collections (e.g. Pasteur culture collection of cyanobacteria, PCC, Paris, France) were isolated from aquatic habitats, but many cyanobacteria are also found in diverse terrestrial habitats such as on soils or surfaces of rocks. Beside nutrients, light and temperature, the availability of water and the amount of dissolved ions (total salinity) are important environmental factors determining the occurrence of strains in specific environments. Because total salinity and water amount are closely linked, e.g. during desiccation of soil, the amount of water is decreasing and, in parallel, the total salt concentration is increasing, it is not surprising that acclimation towards drought and high salinity employs overlapping mechanisms. In both cases, the maintenance of water and turgor pressure inside the cell is one of the central issues during the acclimation (experimentally verified for cyanobacteria by [Ladas and Papageorgiou \(2000\)](#)). Because water uptake is a passive process following the water potential gradient, growing microbial cells need to establish a lower water potential inside the cell relative to the surrounding medium, which is achieved by regulating the cellular osmotic potential via varying amounts of low molecular compounds.

The main difference between pure water or osmotic stress and salt stress is the additional direct ion effect on metabolic activities in the latter case. In the nature exists large variations regarding the amount and composition of inorganic salts, which clearly affects cyanobacterial distribution ([Oren, 2000](#)). In addition to the problem that high total ion content generally makes it difficult to maintain water and turgor inside the cell, many ions are toxic for living cells. This direct toxicity is true not only for heavy metals but also for any ion at nonphysiological high cellular concentrations.

This chapter deals with cyanobacterial salt acclimation in the genomic era. Because the annotation of genome information relies on the knowledge of well-studied model organisms, the author will use here the term high salt equivalent for an enhanced  $\text{Na}^+$  and  $\text{Cl}^-$  contents, the main inorganic ions in the marine environment, which are usually used in laboratory experiments to mimic salt stress.





## 2. BASIC SALT ACCLIMATION STRATEGY

To acclimate to a quick increase in the external salinity or to live permanently in the high salt environment, cyanobacteria as most other cells apply the so-called ‘salt-out’ strategy (Galinski, 1995). The other strategy is called ‘salt-in’ strategy, which is used by some halophilic Archaea and Bacteria. The latter prokaryotes accumulate high internal amounts of inorganic salts (especially KCl) exceeding the external salt concentration. The accumulation of inorganic ions is metabolically cheaper to increase internal osmotic concentrations and to ensure water uptake, turgor pressure and growth. However, the presence of high salt concentrations in the metabolically active compartment needed an adaptation of all organic macromolecules to this new environment, which seemed to be difficult to achieve during evolution. This assumption explains why the energetic favourable ‘salt-in’ strategy is restricted to a few prokaryotes.

In contrast, organisms using the ‘salt-out’ strategy can keep the normal set of low-salt-resistant proteins for metabolic activity but needed to find an energetically more expensive strategy to balance the osmotic potential difference. These organisms are characterized by almost unchanged internal ion concentration after acclimation to high NaCl concentrations. This observation is especially true for amounts of  $\text{Na}^+$  and  $\text{Cl}^-$  (e.g. shown for *Synechocystis* sp. PCC 6714 by Reed, Warr, Richardson, Moore, and Stewart (1985)). To keep the low internal NaCl concentration in the presence of high external salinities,  $\text{Na}^+$  and  $\text{Cl}^-$  are actively pumped out from the cells. Multiple transporters are used for  $\text{Na}^+$  export by cyanobacteria. Predominantly, specific members of the  $\text{Na}^+/\text{H}^+$  antiporter family seem to be involved (Elanskaya, Karandashova, Bogachev, & Hagemann, 2002; Inaba, Sakamoto, & Murata, 2001; Waditee et al., 2001, 2002; Wang, Postier, & Burnap, 2002). Additionally, the Mrp system is used for  $\text{Na}^+$  export (Blanco-Rivero, Leganés, Fernández-Valiente, Calle, & Fernández-Piñas, 2005; Fukaya et al., 2009). These transporters receive the energy for ion export from the proton gradient at the membrane. For many years, it was discussed if cyanobacteria and eukaryotic algae also employ primary active  $\text{Na}^+$ -ATPases for ion export during salt acclimation (Gimmler, 2000). Only recently, direct experimental evidence for the presence and activity of  $\text{Na}^+$ -ATPases of the  $\text{F}_1\text{F}_0$ -type was presented for *Aphanothece halophytica* (Soontharapirakkul et al., 2011) and from genome information for some more



strains like *Acaryochloris marina* (Dibrova, Galperin, & Mulkidjanian, 2010). Compared to  $\text{Na}^+$ , the export of  $\text{Cl}^-$  is much less well understood among cyanobacteria (for a review, see Hagemann (2011)).

Additional to the energy demand for ion export, cells using the ‘salt-out’ strategy need energy and organic matter for the accumulation of high amounts of compatible solutes, which are used instead of inorganic ions to balance the osmotic potential and to maintain turgor. These compounds are low-molecular-mass organic molecules that are highly water soluble and usually do not carry net charge at physiological pH. Compatible solutes can be accumulated in high (molar) amounts without negative interference (i.e. being compatible) towards the metabolisms (Brown, 1976). In addition to the osmotic equilibrium, the compatible solutes can also exhibit direct protective effects towards sensitive macromolecules. The protective effect explains why often the accumulation of rather low amounts of compatible solutes, i.e. at concentrations not making big contribution to the intracellular osmotic potential, results in significant increase of salt or drought stress tolerance (for a review, see Chen and Murata (2011)).



### 3. COMPATIBLE SOLUTES

After the first description of glucosylglycerol (GG) as compatible solute in a marine *Synechococcus* strain (Borowitzka, Demmerle, Mackay, & Norton, 1980), about 200 cyanobacteria were screened for such compounds and their salt-resistance range (see Hagemann (2011) for a comprehensive table). This data set revealed that a rather small spectrum of compatible solutes is found in salt-loaded cyanobacteria, and, that a correlation between the chemical nature of the compatible solute and the salt resistance limits exists (Reed, Borowitzka et al., 1986). Accordingly, group 1 of low salt tolerance accumulates sucrose and/or trehalose (150 examples), group 2 of moderate halotolerance prefers GG (71 examples), and group 3 of halophilic strains have an absolute requirement for a minimal salt concentration and synthesize glycine betaine (22 examples) as characteristic compatible solute. Unfortunately, cyanobacterial taxonomy is problematic. Species names as well as strain numbers have been changed or mixed. Therefore, it is difficult to make a direct comparison of the strain list with compatible solutes (see Hagemann, 2011) and the list of compatible solute genes derived from genome sequences of cyanobacteria (Table 2.1).

In this chapter, the author will often refer to two big groups of cyanobacteria, which were initially distinguished according to their RubisCO

(one carrying RubisCO form I-A or RubisCO form I-B) and carboxy-some types (Hess et al., 2001). Later they were named alpha-cyanobacteria (mostly picoplanktonic oceanic *Prochlorococcus* and *Synechococcus* strains) and beta-cyanobacteria (Badger, Hanson, & Price, 2002). To analyse the molecular basis of compatible solute synthesis, mostly beta-cyanobacterial strains have been investigated: *Nostoc* (*Anabaena*) sp. PCC 7120 (hereafter *Nostoc* 7120) for group 1, *Synechocystis* sp. PCC 6803 (hereafter *Synechocystis* 6803) for group 2, and *A. halophytica* (hereafter *Aphanothece*) for group 3.

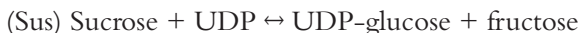
### 3.1. Sucrose

Sucrose ( $\alpha$ -D-glucopyranosyl-(1  $\rightarrow$  2)- $\beta$ -D-fructofuranoside) accumulation was often found in salt-stressed cyanobacteria. Still it is possible to conclude that all heterocystous, N<sub>2</sub>-fixing cyanobacteria use sucrose as major compatible solute at elevated salinity, while generally the preference for a specific compatible solute is not correlated with any other specific cyanobacterial clade (Fig. 2.1). The widespread occurrence of sucrose in salt-stressed cyanobacteria is not surprising because sucrose plays a central role in the carbon metabolism in photoautotrophic organisms (Kolman, Torres, Martin, & Salerno, 2011; Lunn, 2002).

It has been shown that four enzymes are crucial for sucrose accumulation inside photoautotrophic cells. Sucrose-phosphate synthase (Sps) and sucrose-phosphate phosphatase (Spp) are the main sucrose biosynthesis enzymes:



The sucrose synthase (Sus) can catalyse the following reversible reaction; however, it is believed to predominantly degrade sucrose:



Additionally, invertase (sucrase) is known to irreversibly hydrolyse sucrose:



Thus, the activity of sucrose synthesis enzymes relative to the two sucrose degrading enzymes should determine the sucrose steady-state level in cyanobacterial cells (Kolman et al., 2011).

**Table 1** Sixty seven cyanobacterial genomes were searched in GenBank for genes coding enzymes for compatible solute biosynthesis by the BLAST algorithm (Altschul et al., 1997)

Strain	SpsA (SII0045)	SpsA* (Alr3370)	SpsA* (All4376)	Spp (Slr0953)	OtsAB	TreZ
<b>Beta-cyanobacteria</b>						
<i>Acaryochloris marina</i> MBIC11017		YP_001517507.1	YP_001517507.1	YP_001517199.1		
<i>Acaryochloris</i> sp. CCMEE 5410		ZP_09247255.1	ZP_09247255.1	ZP_09249474.1		
<i>Acaryochloris</i> sp. CCMEE 5410		ZP_09251279.1	ZP_09251279.1			
<i>Acaryochloris</i> sp. CCMEE 5410		ZP_09251468.1	ZP_09251468.1			
<i>Anabaena variabilis</i> ATCC 29413		YP_323804.1	YP_323804.1	YP_323329.1		YP_321946.1
<i>Anabaena variabilis</i> ATCC 29413		YP_323913.1	YP_323913.1			
<i>Arthrospira maxima</i> CS-328						ZP_03275188.1
<i>Arthrospira platensis</i> NIES-39						
<i>Arthrospira platensis</i> str. Paraca						ZP_06384104.1
<i>Arthrospira</i> sp. PCC 8005						
Cyanobacterium UCYN-A						
<i>Crocospira watsonii</i> WH 8501					ZP_00516247.1	
<i>Cyanothece</i> sp. ATCC 51142				YP_001802623.1		
<i>Cyanothece</i> sp. CCY0110				ZP_01727894.1		ZP_01730695.1
<i>Cyanothece</i> sp. PCC 7424		YP_002378525.1	YP_002377234.1	YP_002379708.1		YP_002379851.1
<i>Cyanothece</i> sp. PCC 7424		YP_002377234.1	YP_002378525.1			
<i>Cyanothece</i> sp. PCC 7425				YP_002484407.1		
<i>Cyanothece</i> sp. PCC 7425		YP_002482481.1	YP_002482481.1	YP_002485261.1		
<i>Cyanothece</i> sp. PCC 7425		YP_002483625.1	YP_002483625.1			
<i>Cyanothece</i> sp. PCC 7822		YP_003885909.1	YP_003885909.1	YP_003887642.1		YP_003889479.1
<i>Cyanothece</i> sp. PCC 7822		YP_003886096.1	YP_003886096.1			
<i>Cyanothece</i> sp. PCC 8801						YP_002371610.1
<i>Cyanothece</i> sp. PCC 8802						YP_003137180.1
<i>Cylindrospermopsis raciborskii</i> CS-505		ZP_06309461.1	ZP_06309461.1	ZP_06306557.1		
<i>Fischerella</i> sp. JSC-11		ZP_08987552.1	ZP_08987552.1	ZP_08986780.1		ZP_08984802.1
<i>Gleobacter violaceus</i> PCC 7421		NP_926786.1	NP_926786.1	NP_926785.1		
<i>Lyngbya</i> sp. PCC 8106						ZP_01620214.1
<i>Microcoleus chthonoplastes</i> PCC 7420		ZP_05024229.1	ZP_05024229.1	ZP_05024293.1		ZP_05027447.1
<i>Microcoleus chthonoplastes</i> PCC 7420				ZP_05029649.1		
<i>Microcoleus vaginatus</i> FGP-2		ZP_08493316.1	ZP_08493316.1	ZP_08495240.1		ZP_08493057.1
<i>Microcystis aeruginosa</i> NIES-843						

TreY	TreA	GgpS	GgpP	GpgS	GgpgP	GsmT	Dmt
		YP_001516020.1	YP_001516026.1	YP_001517954.1	YP_001517956.1		
		ZP_09250227.1	ZP_09250231.1	ZP_09250357.1	ZP_09250359.1		
YP_321945.1	YP_321944.1						
ZP_03275187.1		ZP_03273530.1	ZP_03275431.1	ZP_03275227.1	ZP_03274421.1		
BAI87980.1		BAI93049.1		BAI91147.1	BAI90985.1		
ZP_06384103.1		ZP_06380107.1	ZP_06380589.1	ZP_06384311.1	ZP_06380864.1		
ZP_09784258.1		ZP_09780739.1	ZP_09781502.1		ZP_09782039.1		
		YP_003421551.1					
		YP_001803806.1	YP_001804705.1				
	ZP_01732618.1	ZP_01726261.1	ZP_01726605.1				
YP_002379850.1							
YP_003889480.1							
YP_002371611.1	YP_002371612.1						
YP_003137181.1							
ZP_08984801.1							
ZP_01620213.1				ZP_01620950.1	ZP_01623921.1	ZP_01618784.1	ZP_01618784.1
ZP_05027675.1		ZP_05025446.1	ZP_05024720.1	ZP_05025802.1	ZP_05030789.1		
ZP_08493056.1							

**Table 1** Sixty seven cyanobacterial genomes were searched in GenBank for genes coding enzymes for compatible solute biosynthesis by the BLAST algorithm (Altschul et al., 1997)—cont'd

Strain	SpsA (Sll0045)	SpsA* (Alr3370)	SpsA* (AII4376)	Spp (Slr0953)	OtsAB	TreZ
<i>Microcystis aeruginosa</i> PCC 7806		CAO88729.1	CAO88729.1	CAO88727.1		
<i>Moorea product</i> 3L		ZP_08427749.1	ZP_08427749.1	ZP_08432285.1		
<i>Moorea product</i> 3L						
<i>Nodularia spumigena</i> CCY9414		ZP_01631316.1	ZP_01631316.1	ZP_01628463.1		ZP_01632239.1
<i>Nodularia spumigena</i> CCY9414	ZP_01629520.1			ZP_01629520.1		
<i>Nostoc azollae</i> 0708		YP_003720706.1	YP_003720706.1	YP_003721505.1		
<i>Nostoc azollae</i> 0708		YP_003722939.1	YP_003722939.1			
<i>Nostoc punctiforme</i> PCC 73102		YP_001865190.1	YP_001865190.1	YP_001866500.1		YP_001868949.1
<i>Nostoc punctiforme</i> PCC 73102		YP_001866499.1	YP_001866499.1	YP_001866908.1		
<i>Nostoc punctiforme</i> PCC 73102		YP_001867882.1	YP_001867882.1			
<i>Nostoc</i> sp. PCC 7120		NP_488416.1	NP_488416.1	CAC43285.1		NP_484212.1
<i>Nostoc</i> sp. PCC 7120		NP_487410.1	NP_487410.1	NP_484420.1		
<i>Oscillatoria</i> sp. PCC 6506						ZP_07112724.1
<i>Synechococcus elongatus</i> PCC 6301	YP_171440.1			YP_171440.1		
<i>Synechococcus elongatus</i> PCC 7942	YP_399827.1			YP_399827.1		
<i>Synechococcus</i> sp. JA-2- 3B'a(2-13)				YP_478310.1		YP_478726.1
<i>Synechococcus</i> sp. JA-3-3Ab				YP_473831.1		YP_473674.1
<i>Synechococcus</i> sp. PCC 7002	AAR31179.1			AAR31179.1		
<i>Synechococcus</i> sp. PCC 7002				YP_001734147.1		
<i>Synechococcus</i> sp. PCC 7335						ZP_05036594.1
<i>Synechocystis</i> sp. PCC 6803	NP_442711.1			NP_441739.1		
<i>Synechocystis</i> sp. PCC 6803				NP_442711.1		
<i>Thermosynechococcus</i> <i>elongatus</i> BP-1	NP_681372.1			NP_681372.1		
<i>Thermosynechococcus</i> <i>elongatus</i> BP-1		NP_682380.1	NP_682380.1			
<i>Trichodesmium erythraeum</i> IMS101						
<b>Alpha-cyanobacteria</b>						
<i>Cyanobium</i> sp. PCC 7001	ZP_05045051.1			ZP_05045051.1		
<i>Cyanobium</i> sp. PCC 7001				ZP_05045012.1		
<i>Prochlorococcus marinus</i> str. AS9601	YP_001010309.1					
<i>Prochlorococcus marinus</i> str. CCMP1375	NP_876271.1					
<i>Prochlorococcus marinus</i> str. CCMP1986	NP_893828.1					
<i>Prochlorococcus marinus</i> str. MIT 9211	YP_001551734.1					
<i>Prochlorococcus marinus</i> str. MIT 9215	YP_001485182.1					

TreY	TreA	GgpS	GgpP	GpgS	GpgpP	GsmT	Dmt
							ZP_08431884.1
							ZP_08430526.1
ZP_01632238.1							
YP_001868950.1	YP_001868951.1						
NP_484211.1	NP_484210.1						
ZP_07112723.1							
YP_478367.1					YP_478588.1		
YP_473881.1					YP_475220.1		
		YP_001736074.1	YP_001736065.1	YP_001735263.1	YP_001735265.1		
ZP_05038218.1	ZP_05036580.1	ZP_05035448.1	ZP_05036760.1				
		NP_441672.1	NP_442928.1				
							YP_722132.1
		ZP_05043859.1	ZP_05046321.1				
			YP_001009088.1	YP_001009287.1	YP_001009289.1		
			NP_875188.1	NP_875119.1	NP_875121.1		
			NP_892757.1	NP_893079.1	NP_893077.1		
			YP_001550632.1	YP_001550559.1	YP_001550561.1		
			YP_001483923.1	YP_001484127.1	YP_001484129.1		

**Table 1** Sixty seven cyanobacterial genomes were searched in GenBank for genes coding enzymes for compatible solute biosynthesis by the BLAST algorithm (Altschul et al., 1997)—cont’d

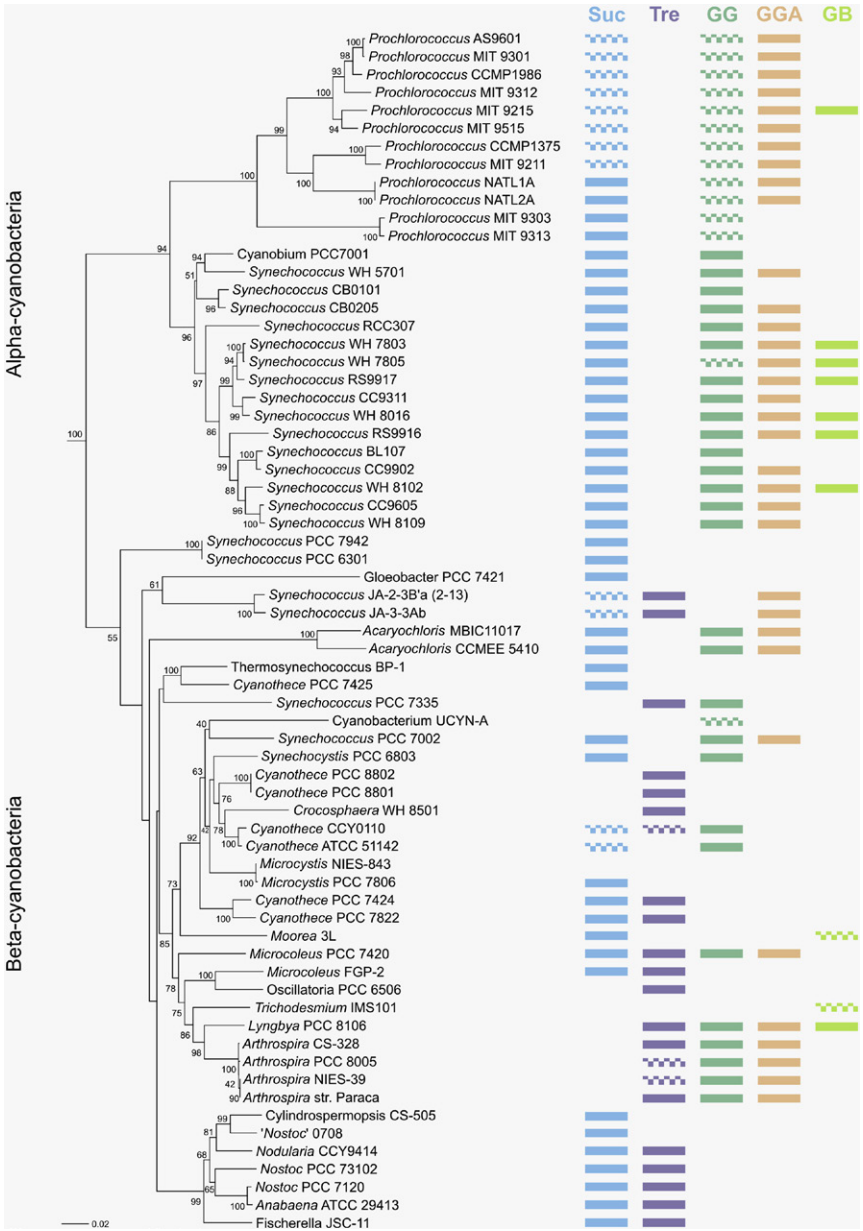
Strain	SpsA (SI0045)	SpsA* (Alr3370)	SpsA* (All4376)	Spp (Slr0953)	OtsAB	TreZ
<i>Prochlorococcus marinus</i> str. MIT 9301	YP_001092125.1					
<i>Prochlorococcus marinus</i> str. MIT 9303	YP_001019012.1			YP_001019012.1		
<i>Prochlorococcus marinus</i> str. MIT 9312	YP_398301.1					
<i>Prochlorococcus marinus</i> str. MIT 9313	NP_896092.1			NP_896092.1		
<i>Prochlorococcus marinus</i> str. MIT 9515	YP_001012216.1					
<i>Prochlorococcus marinus</i> str. NATL1A	YP_001016015.1			YP_001016015.1		
<i>Prochlorococcus marinus</i> str. NATL2A	YP_292514.1			YP_292514.1		
<i>Synechococcus</i> sp. BL107	ZP_01469083.1			ZP_01469083.1		
<i>Synechococcus</i> sp. CB0101	ZP_07974999.1			ZP_07972524.1		
<i>Synechococcus</i> sp. CB0101				ZP_07974999.1		
<i>Synechococcus</i> sp. CB0205	ZP_07969740.1			ZP_07969422.1		
<i>Synechococcus</i> sp. CB0205				ZP_07969740.1		
<i>Synechococcus</i> sp. CC9311	YP_732123.1			YP_732123.1		
<i>Synechococcus</i> sp. CC9605	YP_382969.1			YP_382969.1		
<i>Synechococcus</i> sp. CC9902	YP_378316.1			YP_378316.1		
<i>Synechococcus</i> sp. RCC307	YP_001228785.1			YP_001226627.1		
<i>Synechococcus</i> sp. RS9916	ZP_01471531.1			ZP_01471531.1		
<i>Synechococcus</i> sp. RS9917	ZP_01079206.1			ZP_01079206.1		
<i>Synechococcus</i> sp. WH 5701	ZP_01083582.1			ZP_01083584.1		
<i>Synechococcus</i> sp. WH 5701	ZP_01083584.1			ZP_01083585.1		
<i>Synechococcus</i> sp. WH 5701				ZP_01084261.1		
<i>Synechococcus</i> sp. WH 5701				ZP_01086245.1		
<i>Synechococcus</i> sp. WH 7803	YP_001226250.1			YP_001226250.1		
<i>Synechococcus</i> sp. WH 7805	ZP_01124878.1			ZP_01124878.1		
<i>Synechococcus</i> sp. WH 8016	ZP_08957469.1			ZP_08957469.1		
<i>Synechococcus</i> sp. WH 8102	NP_898609.1			NP_898609.1		
<i>Synechococcus</i> sp. WH 8109	ZP_05789248.1			ZP_05789248.1		

Sucrose-phosphate synthases (SpsA) involved in sucrose biosynthesis were searched with SpsA (SI0045) from *Synechocystis* 6803 (proteins are similar with 2e<sup>-104</sup> or better). Another group of sucrose-phosphate synthases (SpsA\*) involved in sucrose biosynthesis were searched with SpsA\* (Alr3370 or All4376) from *Nostoc* 7120 (proteins are similar with 3e<sup>-72</sup> or better). Sucrose-phosphate phosphatases (Spp) involved in sucrose biosynthesis were searched with Spp (Slr0953) from *Synechocystis* 6803 (proteins are similar with 9e<sup>-9</sup> or better). Maltotoligosyltrehalose synthases (TreY) involved in trehalose biosynthesis were searched with TreY (All0167) from *Nostoc* 7120 (proteins are similar with 7e<sup>-46</sup> or better). Maltotoligosyltrehalose trehalohydrolases (TreZ) involved in trehalose biosynthesis were searched with TreZ (All0168) from *Nostoc* 7120 (proteins are similar with 6e<sup>-113</sup> or better). Glucosylglycerol-phosphate synthases (GgpS) involved in glucosylglycerol biosynthesis were searched with GgpS (SI1566) from *Synechocystis* 6803 (proteins are similar with 3e<sup>-98</sup> or better). Glucosylglycerol-phosphate phosphatases (GgpP) involved in glucosylglycerol biosynthesis were searched with GgpP (Slr0746) from *Synechocystis* 6803 (proteins are

TreY	TreA	GgpS	GgpP	GpgS	GgpgP	GsmT	Dmt
			YP_001090890.1	YP_001091118.1	YP_001091120.1	YP_001017710.1	YP_001017711.1
			YP_001017345.1				
			YP_397136.1	YP_397333.1	YP_397335.1		
			NP_894695.1			NP_894385.1	NP_894384.1
			YP_001011021.1	YP_001011359.1	YP_001011357.1		
			YP_001014527.1	YP_001014736.1	YP_001014738.1		
			YP_291274.1	YP_291440.1	YP_291442.1		
		ZP_01467787.1	ZP_01468168.1	ZP_01469150.1			
		ZP_07973425.1	ZP_07973431.1				
		ZP_07971307.1	ZP_07971313.1	ZP_07970707.1	ZP_07970705.1		
		YP_730607.1	YP_730381.1	YP_729592.1	YP_729594.1		
		YP_381716.1	YP_382080.1	YP_382889.1	YP_382887.1		
		YP_377087.1	YP_376875.1	YP_378245.1	YP_378243.1		
		YP_001227629.1	YP_001227635.1	YP_001228257.1	YP_001228259.1		
		ZP_01472077.1	ZP_01470228.1	ZP_01471834.1	ZP_01471831.1	ZP_01471810.1	ZP_01471811.1
		ZP_01080283.1	ZP_01080399.1	ZP_01080889.1	ZP_01080887.1	ZP_01080893.1	ZP_01080894.1
		ZP_01083670.1	ZP_01083664.1		ZP_01086608.1		
		YP_001224958.1	YP_001225121.1	YP_001226198.1	YP_001226196.1	YP_001224084.1	YP_001224083.1
			ZP_01124278.1	ZP_01124959.1	ZP_01124961.1	ZP_01123473.1	ZP_01123474.1
		ZP_08955132.1	ZP_08954908.1	ZP_08956138.1	ZP_08956136.1	ZP_08956143.1	ZP_08956144.1
		NP_897374.1	NP_896953.1	NP_898525.1	NP_898523.1	NP_898005.1	NP_898004.1
		ZP_05788791.1	ZP_05788970.1	ZP_05789780.1	ZP_05788408.1		

similar with  $8e^{-93}$  or better). Glucosylglycerate-phosphate synthases (GpgS) involved in glucosylglycerate biosynthesis were searched with GggS (SYNPCC7002\_A2021) from *Synechococcus* 7002 (proteins are similar with  $1e^{-96}$  or better). Glucosylglycerate-phosphate phosphatases (GpgP) involved in glucosylglycerate biosynthesis were searched with GgpP (SS120\_Pro0729) from *Prochlorococcus* SS120 (proteins are similar with  $1e^{-11}$  or better). Glycine/sarcosine-*N*-methyltransferases (GSMT) involved in glycine betaine biosynthesis were searched with GSMT (Q33WC4.1) from *Aphanothece* (proteins are similar with  $2e^{-116}$  or better). Dimethylglycine-*N*-methyltransferases (DMT) involved in glycine betaine biosynthesis were searched with DMT (Q83WC3.1) from *Aphanothece* (proteins are similar with  $7e^{-76}$  or better).





**Figure 2.1** Phylogenetic tree (neighbour joining algorithm) of 67 cyanobacterial strains with known genome sequence. The tree is divided into the large groups of alpha- and beta-cyanobacteria. The colour bars represent whether or not genes for compatible solute biosynthesis (Suc – sucrose; Tre – trehalose; GG – glucosylglycerol; GGA – glucosylglycerate; GB – glycine betaine) were found in the genome sequences (see Table 2.1). Dotted bars indicate that the pathway is only incomplete. See the colour plate.

The involvement of Sps in salt-induced sucrose synthesis has been verified for the cyanobacterial strains *Anabaena* sp. PCC 7119 (Porchia & Salerno, 1996), *Nostoc* 7120 (Cumino, Curatti, Giarrocco, & Salerno, 2002), *Synechocystis* 6803 (Curatti et al., 1998; Hagemann & Marin, 1999), and *Synechococcus* sp. PCC 7002 (Cumino, Perez-Cenci, Giarrocco, & Salerno, 2010). These studies revealed that Sps is the sucrose synthesis enzyme among cyanobacteria because purified native or recombinant enzymes showed sucrose synthesis activities, the corresponding transcripts and proteins are accumulated under salt-stress conditions, and *spsA* mutants lost the ability to accumulate sucrose. The cyanobacterial Sps seems to use UDP-glucose rather than ADP-glucose as glucosyl donor, because mutation of the ADP-glucose synthesis enzyme abolished glycogen and GG synthesis but not sucrose accumulation in *Synechocystis* 6803 (Miao, Wu, Wu, & Zhao, 2003).

Searching the presently available cyanobacterial genomes (April 2012) revealed that *spsA* gene can be found in many but not in all cyanobacterial strains (Table 2.1). A closer look shows that at least two different subclasses of Sps proteins exist. First, the Sps protein from *Synechocystis* 6803, which represents a biochemical characterized protein with a biochemically active Sps domain and a biochemically inactive Spp domain (Lunn et al., 1999), was used in BLAST searches (Altschul et al., 1997). These searches indicated that beside *Synechocystis* 6803, only a few other beta-cyanobacteria harbour proteins of high similarity, while all alpha-cyanobacteria encode those Sps proteins in their genomes (Table 2.1). During the analysis of the genetic and biochemical basis for salt-induced sucrose accumulation of heterocystous strains, two genes were identified coding for Sps proteins, which are shorter than the Sps from *Synechocystis* 6803 because the Spp domain is missing (Cumino et al., 2002). Moreover, these proteins showed also lower similarities to SpsA from *Synechocystis* 6803 than sucrose synthases from filamentous strains. This second type of Sps is marked by ★ in Table 2.1. Additional to the *Anabaena/Nostoc* strains, these Sps★ proteins are frequently found in genomes of other beta-cyanobacteria, mostly in two copies. There are only a few strains, e.g. *Nodularia spumigena* CCY9414, harbouring genes for the two Sps types in one genome. Sps★ protein-coding genes seem not to exist in alpha-cyanobacterial genomes.

Interestingly, there are a few cyanobacterial strains with completely known genomes, e.g. *Microcystis aeruginosa* NIES-843 or *Crocospaera watsonii* WH 8501, which are virtually free from any Sps-coding genes. For the latter strains, we showed recently that sucrose is indeed not accumulated under saline conditions (Pade, Compaoré, Klähn, Stal, & Hagemann, 2012). However, whether or not those strains are completely unable to make

sucrose is not known. Alternatively, one can assume that sucrose synthases (Sus), or another member of an uncharacterized glucosyltransferase group, could also be used for sucrose biosynthesis, despite the usual preference of the sucrose hydrolysis reaction. However, at least for the strains *Anabaena* sp. PCC 7119 and *Nostoc* 7120, it has been shown that sucrose synthase plays rather an important role for N<sub>2</sub>-fixation, probably producing precursors for cell wall synthesis or for nitrogen fixation from sucrose (Cumino, Marcozzi, Barreiro, & Salerno, 2007). Similar to *spsA*, *sus* genes are also not universal among cyanobacteria (data not shown here). For example, the genome of *M. aeruginosa* NIES-843 has neither *spsA* nor *sus*.

The second step in sucrose biosynthesis is catalysed by SPP. For this enzyme, separate genes are found in almost all cyanobacterial genomes (Table 2.1). Generally *spsA* containing genomes contain at least one Spp-coding sequence. In most cases, *spsA* and *spp* genes are not linked, however, in *Synechococcus* sp. PCC 7002, they form a salt-regulated operon (Cumino et al., 2010). Interestingly, among *Prochlorococcus* spp., many genomes contain an *spsA* but no *spp* gene (Table 2.1; Scanlan et al., 2009). Whether, in these cases, the C-terminal Spp domain of the SpsA protein is biochemical active or another sugar phosphatase performs the second step in sucrose biosynthesis is not known. It has been experimentally proven that *Prochlorococcus* strains accumulate sucrose as main compatible solute (Klähn, Steglich, Hess, & Hagemann, 2010), therefore, they are obviously able for *de novo* sucrose synthesis and should perform Spp activity.

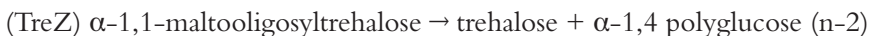
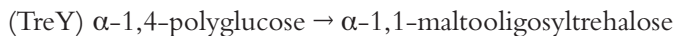
### 3.2. Trehalose

Trehalose ( $\alpha$ -D-glucopyranosyl-(1  $\rightarrow$  1)- $\alpha$ -D-glucopyranoside) is a widespread compatible solute, which not only is protecting against salt but also helps to tolerate heat, dehydration and many other stresses (Furuki, Oku, & Sakurai, 2009). Among cyanobacteria, trehalose is made by two different biosynthetic pathways. The OtsAB pathway was initially elucidated in *Escherichia coli* and employs two enzymes (Strøm & Kaasen, 1993), trehalose-phosphate synthase (OtsA) and subsequently the trehalose-phosphate phosphatase (OtsB) catalysing the following two reactions:



This pathway is used by most bacteria and eukaryotic organisms for stress-induced trehalose biosynthesis. However, in the past years, alternative

trehalose synthesis pathways were found (e.g. Wolf, Krämer, & Morbach, 2003), such as TreYZ. In this pathway, a polysaccharide (i.e. glycogen) precursor is first changed at the terminal end to  $\alpha$  1,1 sugar bound by maltooligosyltrehalose synthase (TreY), and then the trehalose is cleaved off by maltooligosyltrehalose trehalohydrolase (TreZ):



Molecular analyses of cyanobacterial trehalose synthesis revealed that the TreYZ pathway seems to be mostly used. The occurrence of the TreYZ pathway among cyanobacteria was first verified in *Nostoc* 7120 during the study of a gene cluster, which was previously found to be upregulated under desiccation (Higo, Katoh, Ohmori, Ikeuchi, & Ohmori, 2006). In addition to genes for TreY (*all0167*), TreZ (*all0168*), this operon codes for a trehalase (TreA, *all0166*), which is able to hydrolyse trehalose. It should be mentioned that *Nostoc* 7120 accumulates only sucrose under salt stress, while trehalose seems to be made in response to drought stress. Subsequently, the TreYZ pathway has also been found in other trehalose-accumulating cyanobacteria, such as *Nostoc punctiforme* IAM M-15 (Yoshida & Sakamoto, 2009), *Arthrospira platensis* NIES-39 (Ohmori, Ehira, Kimura, & Ohmori, 2009), and *Nostoc flagelliforme* (Wu, He, Shen, Zhang, & Wang, 2010). Using the TreYZA proteins from *Nostoc* 7120 in Blast searches, genes showing high similarities were found in 19 cyanobacterial genomes (Table 2.1). These strains all belong to the group of beta-cyanobacteria; many of them are filamentous and/or N<sub>2</sub>-fixing cyanobacteria, which display often a high-desiccation tolerance (e.g. *Microcoleus vaginatus* FGP-2; Starkenburg et al., 2011). However, genomes of a few unicellular, non-N<sub>2</sub>-fixing strains contain also these genes. Interestingly, the genomes of the two thermophilic *Synechococcus* strains (JA-3-3Aba) from Yellowstone National Park, USA contain TreYZ-coding genes. Most probably, these strains synthesize trehalose as thermoprotectant in their hot environment because this disaccharide exhibits excellent stabilizing activity for macromolecules at high temperatures (Furuki et al., 2009).

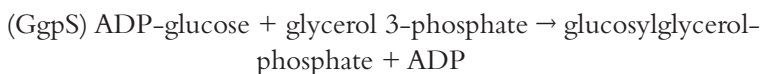
With the only exception *C. watsonii* strain WH 8501, genes coding the *E. coli*-like OtsAB pathway for trehalose synthesis are absent from cyanobacteria (Table 2.1). Only the genome of this unicellular, marine N<sub>2</sub>-fixing cyanobacterium codes for a large fusion protein comprising OtsA and OtsB

domains (Pade et al., 2012). Corresponding to the occurrence of the *otsAB* gene, *Crocospaera* accumulates trehalose as only compatible solute. Overexpression of this gene in *E. coli* verified that the gene codes for a functional trehalose biosynthesis enzyme. Interestingly, *C. watsonii* WH 8501 acquired this gene by a lateral gene transfer event (Pade et al., 2012). Cyanobacteria closely related to *Crocospaera* usually accumulate GG, however, these genes are missing (lost?) in this cyanobacterium.

### 3.3. Glucosylglycerol

Glucosylglycerol (GG;  $\alpha$ -D-glucopyranosyl-(1  $\rightarrow$  2)-glycerol) was the first compatible solute, whose accumulation was found in a cyanobacterium (Borowitzka et al., 1980). Later on, it was detected in many cyanobacterial strains and was regarded to be characteristic for the group of moderate halotolerance (Hagemann, 2011; Reed, Borowitzka et al., 1986). While GG-accumulating beta-cyanobacteria are mostly euryhaline, i.e. they can be cultivated in freshwater and salt-containing media (e.g. *Synechocystis* 6803 or *Synechococcus* sp. PCC 7002; Engelbrecht, Marin, & Hagemann, 1999; Marin, Zuther, Kerstan, Kunert, & Hagemann, 1998), the GG-accumulating picoplanktonic *Synechococcus* strains, which belong to the alpha-cyanobacteria, show only a small range of halotolerance, i.e. they can be cultivated only in media near the normal seawater salinity level (Klähn, Steglich et al., 2010). Beside its osmotic function, GG has good direct membrane and protein stabilizing properties, which are also of biotechnological interest (Borges, Ramos, Raven, Sharp, & Santos, 2002; Hinch & Hagemann, 2004; Sawangwan, Goedl, & Nidetzky, 2010).

Cyanobacteria use a two-step biosynthesis for GG (Hagemann & Erdmann, 1994) with an initial GG-phosphate synthase (GgpS) and a subsequent GG-phosphate phosphatase (GgpP):



This biosynthetic pathway is similar to that of sucrose, trehalose and glucosylglycerate (Klähn & Hagemann, 2011). However, sucrose and trehalose biosyntheses prefer UDP-glucose as glucosyl donor, while cyanobacterial GG synthesis is strictly dependent on ADP-glucose (Hagemann & Erdmann, 1994; Miao et al., 2003). Many heterotrophic bacteria also accumulate GG

under saline conditions. These organisms use ADP- and also UDP-glucose for the GgpS reaction. Moreover, in many heterotrophic bacteria, the two enzyme activities, GgpS and GgpP, are found in one continuous protein (Hagemann et al., 2008). Such fused GG synthesis proteins have not yet been found in any cyanobacterial genome.

Genome searches using the GgpS protein (Sl1566) from *Synechocystis* 6803 (Marin et al., 1998) identified 28 genes coding for highly similar proteins in other cyanobacteria (Table 2.1). The closest homologue is the GgpS from *Synechococcus* sp. PCC 7002, which has been functionally verified (Engelbrecht et al., 1999). Among beta-cyanobacteria, it is also found in many euryhaline and marine strains such as *Arthrospira* (Yoshikawa et al., 2011), *Acaryochloris*, and some but not all *Cyanothece* strains (Table 2.1). However, there are also many marine strains, which do not harbour *ggs* genes in their genomes and are accordingly not using GG as main compatible solute. Interestingly, the marine mat-forming *Microcoleus chthonoplastes* contains genes for GG synthesis, whereas the terrestrial *M. vaginatus* strain FGP-2 is only able to synthesize trehalose (Table 2.1). Probably, in the terrestrial habitat, desiccation is the main stress, which is usually tolerated by trehalose accumulation, while the marine *Microcoleus* is faced by salt stress, therefore preferring GG.

As previously reported (Scanlan et al., 2009), practically all of the marine picoplanktonic *Synechococcus* strains (only exception is *Synechococcus* sp. WH 7805) contain *ggs* genes, whereas the related *Prochlorococcus* strains miss it all (Table 2.1). The accumulation of GG in marine *Synechococcus* and its absence in *Prochlorococcus* strains have been recently verified (Klähn, Steglich et al., 2010).

The GgpP (StpA was used synonymously) protein is a specific GG-phosphate phosphatase. Homologues of this protein are restricted to cyanobacteria and are characterized by a specific protein domain called Salt\_tol\_Pase superfamily. The only functional characterized GgpP enzyme is encoded by *slr0746* in *Synechocystis* 6803 (Hagemann, Schoor, Jeanjean, Zuther, & Joset, 1997). Highly similar proteins are found in all cyanobacterial genomes harbouring a *ggs* gene. Among beta-cyanobacteria *ggs* and *ggsP* never form an operon. However, in the oceanic picoplanktonic *Synechococcus* strains, these genes coding for functional linked proteins are often found adjacent to each other indicative for operon structures.

In one case, *Synechococcus* sp. RCC307, not only *ggs* and *ggsP* are linked but also the four subunits for the putative ABC-type GG-transport system

*ggtABCD* are situated downstream in the genome (Scanlan et al., 2009). Because a clear functional assignment of ABC transporters by sequence similarity searches is difficult, the occurrence of GG-transporter genes as well as genes for other compatible uptake systems was not analysed here. Generally, it can be assumed that at least transporters for their own main compatible solute are present among cyanobacteria, which prevent leakage of compatible solutes in the medium as has been shown for *ggtA* mutant of *Synechocystis* 6803 (Hagemann, Richter, & Mikkat, 1997; Mikkat, Effmert, & Hagemann, 1997).

Surprisingly, *gppP* genes are also present in the *Prochlorococcus* genomes, which do not contain *gppS* genes. Since the only known biochemical function of GgpP is dephosphorylation of the intermediate GG-phosphate, one can speculate that the common ancestor of alpha-cyanobacteria harboured *gppS* and *gppP* genes (Scanlan et al., 2009). Early in the evolution of the *Prochlorococcus* clade, the *gppS* gene was lost and sucrose replaced GG as major compatible solute. Why the *gppP* gene was kept is uncertain, possibly the GgpP is able to dephosphorylate also other sugar phosphates. An interesting possibility would be that in *Prochlorococcus* strains, the GgpP may act as sucrose-phosphate phosphatase, because separate *spp* genes are missing from all *Prochlorococcus* genomes (Table 2.1).

### 3.4. Glucosylglycerate

Glucosylglycerate (GGA) is an uncommon compatible solute because it carries a net charge at physiological pH. GGA has been early detected in extracts of the cyanobacterium *Synechococcus* sp. PCC 7002 (*Agmenellum quadruplicatum*) (Kollman, Hanners, London, Adame, & Walker, 1979). GGA and its structural relative mannosylglycerate have been extensively analysed in thermophilic, heterotrophic bacteria (Empadinhas & da Costa, 2008). The identification of the structural genes for GGA synthesis revealed that genes for similar proteins occur also in cyanobacterial genomes (Costa et al., 2006). The biosynthetic pathway resembles that of GG, sucrose and trehalose, a GGA-phosphate synthase (GpgS) cooperates with a phosphatase (GpgP):

(GpgS)  $\text{NDP-glucose} + \text{glycerate 3-phosphate} \rightarrow \text{GGA-phosphate} + \text{NDP}$

(GpgP)  $\text{GGA-phosphate} \rightarrow \text{GGA} + \text{Pi}$

Recently, we could confirm that GGA is used as compatible solute among cyanobacteria (Klähn, Steglich et al., 2010). GGA accumulation was found

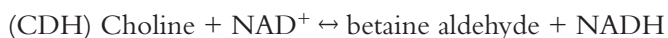


in many alpha-cyanobacteria, i.e. *Prochlorococcus* and *Synechococcus* spp., and in the beta-cyanobacterium *Synechococcus* sp. PCC 7002. An interesting observation was that the GGA amount was not only dependent from the salinity level; its accumulation became clearly stimulated or even induced when salt addition was combined with nitrogen limitation. Especially, the *Synechococcus* strains were virtually free of GGA under N-excess but contained high internal amounts at N-limited growth (Klähn, Steglich et al., 2010). This finding gave rise to the hypothesis that the charged compatible solute GGA is replacing glutamate under N-limiting conditions and serve as organic counterion for cations, especially  $K^+$ , inside the salt-loaded cells of marine cyanobacteria, which are usually faced by N-limiting conditions.

Overexpression of the *gpgS* gene from *Synechocystis* sp. PCC 7002 allowed the purification of recombinant GpgS protein, which showed the expected biochemical activity. Similar proteins are coded in the genomes of most alpha-cyanobacteria (only two *Prochlorococcus* and two *Synechococcus* strains miss *gpgS* genes, Table 2.1), while only a few beta-cyanobacterial genomes code for this enzyme. As in heterotrophic bacteria, in alpha-cyanobacteria, the *gpgS* genes are usually found in an operon with two other genes coding further proteins for GGA metabolism, i.e. GGA hydrolase and GpgP. Genes for GpgP (mostly wrongly annotated as mannosyl-3-phosphoglycerate phosphatase in cyanobacterial genomes) are found in almost all cyanobacterial genomes with *gpgS* genes (only exception is *Synechococcus* sp. BL107). There are few genomes (e.g. the two thermophilic *Synechococcus* strains from Yellowstone National Park) carrying the *gpgP* but no *gpgS* gene, which could be taken as an indication that the GGA biosynthesis became stepwise lost in these strains.

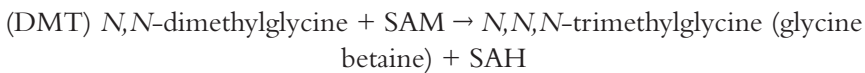
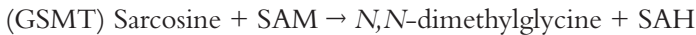
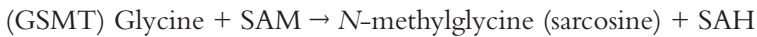
### 3.5. Glycine Betaine

Glycine betaine (*N,N,N*-trimethylglycine) is a widespread compatible solute, which has been reported from many salt-stressed organisms (for a review, see Chen and Murata (2011)). In most organisms, glycine betaine is synthesized by a two-step oxidative pathway using choline as precursor, in which choline dehydrogenase (CDH) encoded by *betA* and betaine aldehyde dehydrogenases (BADH) encoded by *betB* in *E. coli* (Andresen, Kaasen, Styrvoid, Boulnois, & Strøm, 1988) are cooperating.





Later on, genes and proteins for alternative glycine betaine synthesis were identified in heterotrophic bacteria (Nyyssola, Kerovuo, Kaukinen, von Weymarn, & Reinikainen, 2000). This pathway is performed by two enzymes, glycine/sarcosine-*N*-methyltransferase (GSMT) and then dimethylglycine-*N*-methyltransferase (DMT), which catalyse the three-step methylation of glycine with the methyl-donor *S*-adenosyl-methionine (SAM):



The direct methylation of glycine seems to be used by all glycine-betaine-accumulating cyanobacteria. Its activity has been verified in the hypersaline model strain *Aphanothece* and in the picoplanktonic *Synechococcus* sp. WH 8102 (Lu, Chi, & Su, 2006; Waditee et al., 2003, 2005). Screening the database with the GsmT and Dmt sequences from *Aphanothece*, which itself is not completely sequenced, showed only a small number of highly similar proteins in cyanobacterial genomes. Among beta-cyanobacteria, only *Lynbya* sp. PCC 8106 and a related newly described species *Moorea product* 3L (Engene et al., 2012), which was isolated from seagrasses, contain these genes for glycine betaine synthesis. The filamentous  $\text{N}_2$ -fixing *Trichodesmium erythraeum* IMS101 harbours only a Dmt-coding gene in its chromosome, while the GsmT sequence is missing. In the moment, it is unknown which compatible solute is accumulated in this marine strains. Our own screenings of salt-loaded cells of *Trichodesmium* did not detect any glycine betaine but another, yet unknown compound in osmotic significant concentrations (Hagemann et al., unpublished results). Among the alpha-cyanobacteria, genes coding for GsmT and Dmt are found in eight *Synechococcus* or *Prochlorococcus* genomes. These strains contain also genes for many other compatible solute biosynthesis enzymes and seem to be rather euryhaline and able to live in dynamic ecosystems (Mao et al., 2010; Scanlan et al., 2009).

Compared to the number of strains accumulating other compatible solutes, a rather low number of glycine betaine accumulators can be predicted from the genome information. One explanation could be that cyanobacteria can also use the oxidative *E. coli*-like BetAB pathway for glycine betaine synthesis. Using the BetAB sequences in genome searches is not very

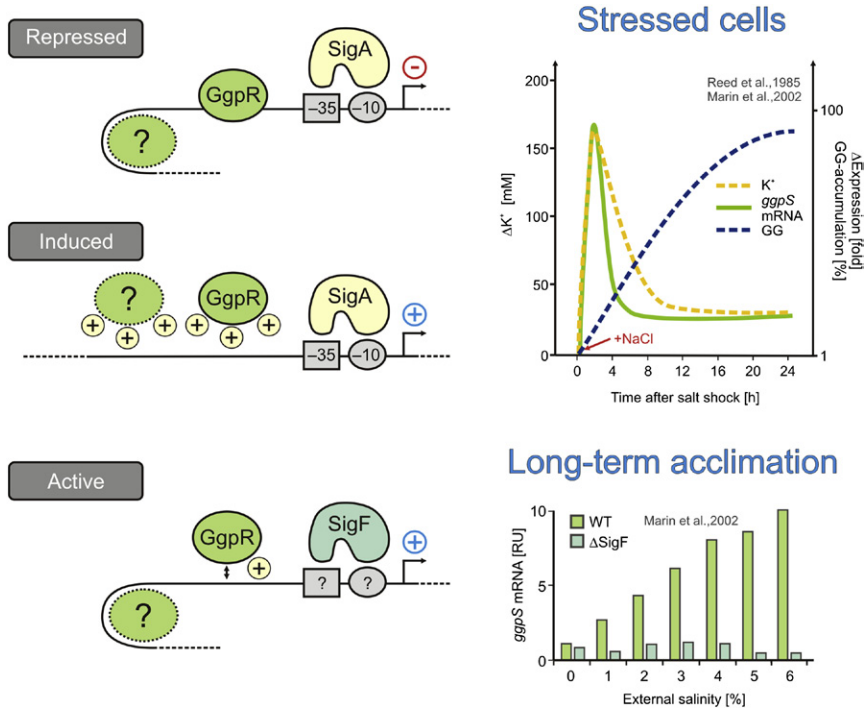
informative since many noncharacterized aldehyde oxidase/dehydrogenases display a certain degree of similarity. However, those proteins are also frequently found in genomes of strains, which definitely do not accumulate glycine betaine. In the moment, it seems to be more likely that cyanobacteria use exclusively the methylation pathway for glycine betaine synthesis and the oxidative pathway is not active. This view is indirectly supported by the results of two studies, which introduced the oxidative glycine betaine pathway into the freshwater strain *Synechococcus* sp. PCC 6301 (Deshnium, Los, Hayashi, Mustardy, & Murata, 1995; Nomura, Ishitani, Takabe, Rai, & Takabe, 1995). Significant glycine betaine accumulation was only detected when the medium was supplemented with the BetAB pathway precursor choline, which seems to be limiting in cyanobacteria, whereas glycine as precursor for the methylation pathway is available in high amounts.



## 4. REGULATION

Compatible solute synthesis needs to be regulated according to the external salt conditions, i.e. the cellular concentration of compatible solutes varies according to the amount of external total salts. There are many examples showing that transcripts for compatible solute biosynthesis genes increase after salt-shock treatments (e.g. *ggs* for GG synthesis, Marin, Huckauf, Fulda, & Hagemann, 2002; *sps* for sucrose biosynthesis, Cumino et al., 2010). In most cases, the transcription is highly stimulated after the salt-shock treatment, in the case of *ggs* up to 50-fold, whereas the final steady-state contents are only slightly elevated and depend on the external salt concentration (Fig. 2.2). Interestingly, the transcript amounts of glucosylglycerate biosynthesis genes showed a rather small increase after salt addition, which became much more pronounced and extended when salt-stress treatments were done under N-limiting conditions (Klähn, Steglich et al., 2010).

The sensing of the salt-stress signal and its transduction to the gene expression is less good understood. Many potential signals are discussed to inform the cell about the external salinity (Wood, 1999). Intracellular inorganic ions or turgor changes are among those possible signals. In the case of *E. coli*, a transient rise in  $K^+$  amounts, which has been also observed in salt-shocked cells of *Synechocystis* sp. PCC 6714 (Reed et al., 1985), changes the promoter-binding specificity of the RNA polymerase towards promoters for genes activated after salt shocks (Gralla & Vargas, 2005). In cyanobacteria, a GAF-domain-containing adenylate cyclase has been characterized as sodium sensor (Cann, 2007). Moreover, knocking out the water channel



**Figure 2.2** Model of transcriptional regulation of the *ggpS* gene for glucosylglycerol (GG) synthesis (left panels) in *Synechocystis* 6803 in comparison to experimental data (right panels). In low-salt grown cells, the *ggpS* gene is repressed leading to GG-free cells. Salt-shock treatments induce the highest *ggpS* expression and quick GG accumulation because the influx of inorganic ions releases the repressor GgpR from the *ggpS* promoter in salt-stressed cells. In long-term salt-acclimated cells, the *ggpS* expression remains active but depends on the external salinity. Moreover, the *ggpS* expression in salt-acclimated cells seems to be driven by SigF instead of SigA because a SigF deletion abolishes *ggpS* expression to a large extent. Possibly, additional regulatory factors (marked by ?) are also involved. See the colour plate.

AqpZ affected not only water flow and turgor, but also the expression of many salt-regulated genes was changed as well in *Synechocystis* 6803 (Shapiguzov et al., 2005).

Analyses of the impact of mutations of two component systems in *Synechocystis* 6803 on salt-regulated gene expression revealed that defined groups of these genes are controlled by pairs of histidine kinases and their cognate response regulators (Marin et al., 2003; Shoumskaya et al., 2005). However, none of the genes involved in GG synthesis or transport showed changes in the salt regulation in any of these mutants. Other candidates for regulating

salt-stress-stimulated gene expression are alternative sigma factors. A mutation of the group 3 sigma factor SigF resulted in the decreased expression of many salt-stress proteins including GgpS (Huckauf, Nomura, Forchhammer, & Hagemann, 2000; Marin et al., 2002) (Fig. 2.2). Moreover, knocking out genes for group 2 sigma factors (especially SigB) also lead to decreased salt tolerance in *Synechocystis* 6803 (Nikkinen et al., 2012). The bioinformatic analysis of the genome sequence and the analysis of salt-regulated transcriptional changes resulted in the prediction that the alternative sigma factor  $\sigma 38$  seems to control the salt-stress-related gene expression pattern in the alpha-cyanobacterium *Synechococcus* sp. WH 8102 (Mao et al., 2010).

A transcriptional factor specifically regulating compatible solute biosynthesis genes or other groups of salt-induced genes is not known among cyanobacteria. The promoter of the salt-regulated *ggpS* gene was mapped in *Synechocystis* 6803. This study revealed that the *ggpS* expression is negatively regulated. An ORF for a small protein was discovered in the *ggpS* promoter region, which codes for GgpR repressing *ggpS* under low salt conditions (Klähn, Höhne, Simon, & Hagemann, 2010). The binding of GgpR to the *ggpS* promoter region is influenced by the concentration of inorganic ions (Klähn et al., unpublished results). This finding lead to the model that the transient accumulation of  $K^+$  in salt-shocked *Synechocystis* 6803 cells releases the GgpR protein from the *ggpS* promoter resulting in its maximal activity. Under steady-state conditions, when the  $K^+$  content decreased, GgpR is loosely associated to the *ggpS* promoter and alternative sigma factors (e.g. SigF) guarantee a stress-proportional *ggpS* expression (Fig. 2.2). Because *ggpR* mutations did not completely abolish the salt regulation of *ggpS*, the involvement of additional regulatory molecules is possible (marked by ? in Fig. 2.2). Interestingly, a small ORF, similar to *ggpR*, also exists upstream of the *ggpS* gene of *Synechococcus* sp. PCC 7002 (Klähn, Höhne et al., 2010).

Additional to transcriptional regulation, compatible solute biosynthesis is tightly regulated on biochemical level. A reversible activation and inactivation of the GgpS activity by addition of high salt and removal of inorganic ions, respectively, has been early described (Hagemann & Erdmann, 1994). It took almost 20 years to elucidate the molecular mechanism. Novak, Stirnberg, Roenneke, and Marin (2011) discovered a new biochemical switch to activate or inactivate GgpS activity. It was found that GgpS can bind DNA or RNA in a sequence-unspecific manner, which switches off the enzyme activity. In the presence of increasing concentrations of inorganic ions, particularly  $K^+$ , the GgpS is released and becomes increasingly active. The model explains why GgpS is maximally active in salt-shocked cells

when the internal ion content is high, whereas its steady-state activity corresponds to the external salinity, because the steady-state amount of internal  $K^+$  increases gradually in cells acclimated to increasing salinities (Novak et al., 2011; Reed et al., 1985).



## 5. CYANOBACTERIAL BIOTECHNOLOGY AND SALT ACCLIMATION

Due to environmental concerns and the future decline in oil production, cyanobacteria are increasingly investigated as alternative sources for green energy and chemical feedstock (e.g. Ducat, Way, & Silver, 2011). To minimize competition with agricultural food production, the mass cultivation of cyanobacteria should be done on land not suited for agriculture and by using salt water instead of freshwater. For this purpose, it is important to gain more knowledge on salt acclimation of a broader set of cyanobacteria. Future production strains will be certainly optimized regarding salt-tolerance mechanisms, i.e. by engineering the energetic cheapest strategy to minimize negative impacts on product biosynthesis.

Compatible solutes will be not only important for enabling high growth rates of production strains in salty media, these chemicals itself are of relatively high value, because they can be used in cosmetics and pharmaceuticals as moisturizers and stabilizers. Presently, GG (Sawangwan et al., 2010) but particularly ectoine, a compatible solute from halobacteria, is produced by the BITOP GmbH (<http://www.bitop.de/cms/website.php?id=/en/index.htm>). The compatible solute ectoine is harvested by a so-called bacterial milking process. It has been previously shown that the alternative incubation in salt medium (production phase) and subsequent incubation in distilled water (harvesting phase) could also be used to elute compatible solutes from cyanobacteria (Fulda, Hagemann, & Libbert, 1990; Reed, Warr, Kerby, & Stewart, 1986). Recently, *Synechococcus* sp. PCC 7942 strains have been engineered, which express a sucrose export system. These strains did not only continuously excrete sucrose into the culture supernatant when grown in saline medium, the overall sucrose biosynthesis and photosynthesis rate were also increased (Ducat, Avelar-Rivas, Way, & Silver, 2012).

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# Iron in Cyanobacteria

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## Abstract

Approximately 40% of global photosynthesis is conducted by phytoplankton in aquatic environments. Cyanobacteria, Gram-negative photoautotrophic prokaryotes, contribute

significantly to this fraction and require large amounts of the essential micronutrient iron in order to maintain their Fe-rich photosynthetic apparatus. Cyanobacterial iron requirements exceed non-photosynthetic prokaryotes by ~10-fold and are exceptionally high even among other photosynthetic organisms. The genomes of cyanobacterial species code for a multitude of iron transporters, iron storage complexes and iron-responsive elements involved in maintaining homeostasis in a highly variable environment. In this chapter, we will review iron transport strategies, the maintenance of intracellular homeostasis and iron limitation responses of cyanobacteria while taking into account the chemistry and environmental bioavailability of iron species.



## 1. IRON BIOGEOCHEMISTRY IN WATER BODIES

### 1.1. Iron Chemistry and the Evolution of Oxygen Evolution

Iron is the fourth most abundant element in Earth's crust, yet Fe bioavailability has been shown to limit primary productivity in large regions of the ocean as well as in many freshwater environments (Boyd *et al.*, 2007). This is due to both Fe concentration and chemistry. In aqueous solutions, iron has two environmentally relevant oxidation states: Fe(II) and Fe(III) (Frausto da Silva & Williams, 2001). Before the evolution of oxygenic photosynthesis, reducing environmental conditions resulted in iron existing primarily in its reduced, lower valency form, Fe(II). Fe(II) is relatively soluble at the circumneutral pH range and, therefore, considered readily bioavailable. It is thus likely that the iron-rich photosynthetic electron transport chain evolved between ~2.3 and 2.2  $10^9$  years ago during the Proterozoic era when Fe(II) was in abundance (Falkowski, 2006). Upon the evolution of oxygenic photosynthesis, molecular oxygen buildup in the atmosphere led to the oxygenation of aquatic environments (Bekker *et al.*, 2004; Allen & Vermaas, 2001). As a result, Fe(II) species were no longer thermodynamically stable and Fe(II) was rapidly oxidized to Fe(III). Relative to its ferrous counterpart, Fe(III) is poorly soluble at the circumneutral pH range (with concentrations of 0.08–0.2 nM in seawater; Liu & Millero, 2002) and precipitates out of solution as ferric oxyhydroxides which are not considered bioavailable (e.g. Rich & Morel, 1990).

Nonetheless, dissolved iron concentrations (where dissolved refers to the fraction passing through a 0.2  $\mu\text{m}$  or 0.45  $\mu\text{m}$  filter) are higher than expected because of complexation by organic ligands which maintain Fe in solution (Fig. 3.1A). It was demonstrated that over 99% of the dissolved Fe(III) in the ocean and freshwater environments is bound by a heterogeneous pool of organic ligands, buffering picomolar equilibrium concentrations of free inorganic Fe(III) species (Nagai, Imai, Matsushige, Yokoi, &

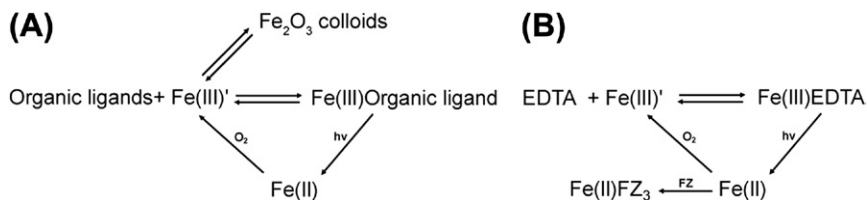


Figure 3.1 Fe speciation chemistry.

Fukushima, 2004; Rue & Bruland, 1995; Wu & Luther, 1995). These organic ligands may be classified according to their conditional stability constants with respect to  $\text{Fe(III)}$  – the stronger L1 class and the weaker L2 class (e.g. Gledhill & van den Berg, 1994). A common view is that L1 is composed of siderophore (strong Fe-specific chelators secreted by microorganisms discussed in further detail in Sections 2.1 and 2.2)–like compounds, whereas L2 is made up of cell degradation products. However, a more complex picture is now emerging with the understanding that many compounds with Fe-binding abilities are released into the water via active secretions, grazing and cell lysis – contributing to an ‘Fe-ligand soup’ in aquatic environments (Hunter & Boyd, 2007). Because these compounds are often found in  $10^3$ – $10^5$  times higher concentrations than iron, they affect the composition of the Fe pool significantly, even though their binding constants are not as high as those of siderophores.

Fe speciation must also be taken into account in laboratory work. Inorganic Fe species (e.g.  $\text{FeCl}_3$ ) will precipitate out of solution as Fe hydroxides whose speciation and stability fluctuate over time. Therefore, in order to work with well-defined Fe substrates as well as with known dissolved iron concentrations,  $\text{Fe(III)}$  must be chelated before use (Fig. 3.1B and Kranzler, Lis, Shaked, & Keren, 2011).

## 1.2. The Iron Hypothesis

Dissolved iron concentrations in many aquatic environments are in the nanomolar to picomolar range (Johnson, Gordon, & Coale, 1997). Large regions of the ocean termed ‘high-nitrate low-chlorophyll’ (HNLC) regions are characterized by sufficient macronutrient concentrations but low chlorophyll concentrations. These regions are also characterized by picomolar levels of dissolved Fe (Martin, Gordon, Fitzwater, & Broenkow, 1989). This observation led to John Martin’s Iron Hypothesis, which suggested that photoautotrophic growth in these large regions of the ocean is in fact limited by low Fe availability (Martin et al., 1994). Numerous iron

enrichment experiments in HNLC waters have supported this hypothesis, showing enhanced phytoplankton growth following iron supplementation (de Baar *et al.*, 2005; Martin, Gordon, & Fitzwater, 1991). These phytoplankton blooms were characterized by an improvement in chlorophyll fluorescence parameters within 24 h, followed by increases in photosynthetic productivity and chlorophyll concentrations. Microscopic analysis of an equatorial Pacific iron fertilization experiment revealed that among the organisms to dominate the bloom was the unicellular cyanobacterium, *Synechococcus* (Martin *et al.*, 1994). Subsequent research has demonstrated that iron bioavailability controls primary productivity in as much as half of the world's ocean (Boyd & Ellwood, 2010). These findings raise questions not only about Fe concentrations but also about the bioavailability of various Fe species within aquatic environments.

### 1.3. Forms of Bioavailable Iron

The bioavailability of iron in both marine and freshwater ecosystems is currently under extensive research (Shaked & Lis, 2012). In many aquatic environments, the iron pool is made up of a heterogeneous mixture of Fe compounds including unchelated inorganic Fe species and organically complexed iron, both of which comprise colloidal/particulate ( $>0.2\ \mu\text{m}$ ) and dissolved fractions ( $<0.2\ \mu\text{m}$ ). Given that the half-life of free ferrous iron in seawater at  $\text{pH} = 8$  is several minutes long (Millero, Sotolongo, & Izaguirre, 1987), free iron is predominantly composed of the ferric iron species ( $\text{Fe}(\text{OH})^{2+}$ ,  $\text{Fe}(\text{OH})_3$  and  $\text{Fe}(\text{OH})^{4-}$ ). This unchelated iron pool will be referred to as  $\text{Fe}'$ ;  $\text{Fe}'$  being the sum of all inorganic iron species in solution (Fig. 3.1). While unchelated dissolved Fe species are the most bioavailable (Hudson & Morel, 1989; Morel, Kustka, & Shaked, 2008), low ambient concentrations cannot support phytoplankton growth. The bioavailability of the organically complexed Fe fraction is the subject of extensive research. Fe ligands such as saccharides have been shown to increase Fe bioavailability to a number of model diatoms (Hassler, Schoemann, Nichols, Butler, & Boyd, 2011). As far as siderophores are concerned, stability constants, midpoint redox potentials, and formation kinetics vary from one species to another resulting in drastic variation in bioavailability. For example, the stability constant of ferrated ferroxamine B (DFB) is  $K = 10^{30}$  (Martell and Smith, 1975) while ferrated aerobactin is  $K = 10^{27.6}$  (Kupper, 2006). Some organisms have been shown to access siderophore-bound iron via reductive processes outside the cell which release the iron before transport. This pathway has been demonstrated for marine prokaryotic and eukaryotic phytoplankton

(Kranzler et al., 2011; Lis & Shaked, 2009; Maldonado & Price, 2001). Other organisms – to date exclusively freshwater autotrophs – are known to transport the entire Fe siderophore into the cell where it undergoes decomplexation.

Particulate and colloidal iron is present in both organic and inorganic forms. Although not classically considered bioavailable in and of themselves, colloidal dissolution can replenish supplies of Fe' and organically complexed iron (Fig. 3.1). This may occur by means of thermal or photochemical processes (Rich & Morel, 1990) and can be siderophore mediated (Kraemer, 2004). Whether colloid dissolution actually occurs is a function of colloid structure and thermodynamic stability (Wells et al., 1983). Recently, work with the filamentous, dinitrogen fixing cyanobacterium, *Trichodesmium*, demonstrated that while only dissolved iron is transported, this organism facilitates dissolution of iron oxides and dust (Rubin, Berman-Frank, & Shaked, 2011). This dissolution was most effectively accomplished by puff-shaped colonies, surrounding dust particles where active shuttling of particulate iron by these colonies was documented (Rubin et al., 2011).



## 2. IRON UPTAKE

### 2.1. Siderophores in Cyanobacteria

Siderophores are the strongest Fe(III) chelators secreted by microorganisms and plants. Siderophore production and secretion occurs, especially under iron starvation, when the intracellular iron concentration drops under a certain threshold required for functionality. Depending on the chemical nature of the organic ligand that coordinates iron, siderophores can be divided into three main classes, the catecholates, the hydroxamates or mixed-types that contain another iron complexing group such as  $\alpha$ -hydroxy-carboxylate next to the hydroxamate or catecholate group (Fig. 3.2; Miethke & Marahiel, 2007). Once bound to Fe(III), the

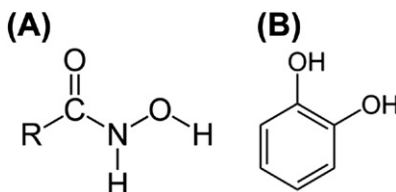


Figure 3.2 Building blocks for iron binding molecules.

ferrisiderophore is transported back into the host cell via specific transporters on the cell surface (See section 2.3).

The secretion of siderophores was first established by a chrome azurol sulfonate (CAS) assay (Schwyn & Neilands, 1987). This assay is based on the transfer of ferric iron complexed with CAS to siderophores, which is accompanied by a shift from blue to yellow. To test for respective hydroxamate- and catecholate-type siderophores, colorimetric measurements were developed by Arnow (1937) and Csaky (1948) methods that are still widely used today. The tyrosinase from the tiger moth *Arctia caja* converts catecholate siderophores to melanin, which can be determined colorimetrically (Arnow, 1937). Similarly, hydroxamates can be oxidized to nitrite in an acetic environment and nitrite can be visualized by means of a colour reaction with sulphanilic acid and  $\alpha$ -naphthylamine (Csáky *et al.*, 1948).

In the past 60 years, many cyanobacteria were tested for their ability to produce and to utilize siderophores. Estep, Armstrong, and van Baalen (1975) as well as Murphy, Lean, and Nalewajko (1976) pioneered the work by demonstrating that siderophores, known to be secreted by other bacteria, can also be detected after growing cyanobacteria in iron-depleted media. McKnight and Morel (1979) could measure hydroxamate-type siderophores in seven different species. The more complex catecholate-type chelator was originally isolated from *Oscillatoria tenuis* (Brown & Trick, 1992). Wilhelm and Trick (1994) demonstrated that hydroxamate-type as well as catecholate-type siderophores can be found in the supernatant of growing cyanobacteria cultures (Table 3.1). Thus, different cyanobacterial classes and species produce a variety of hydroxamate-type siderophores (Goldman, Lammers, Berman, & Sanders-Loehr, 1983).

The hydroxamate-type siderophore schizokinen is a derivative of citric acid and chelates iron via two  $\alpha$ -hydroxamate groups and one  $\alpha$ -hydroxy-carboxylate group (Fig. 3.3A; Simpson & Neiland, 1976). It was initially characterized in the Gram-positive bacterium *Bacillus megaterium* (Mullis, Pollack, & Neilands, 1971) and afterwards in *Anabaena* sp. PCC 6411 (Simpson & Neiland, 1976) and *Anabaena* sp. PCC 7120 (Lammers & Sanders-Loehr, 1982). The function of schizokinen is thought to be twofold. On the one hand, it is part of the iron acquisition strategy (in *Anabaena* sp. PCC 7120, for example, Lammers & Sanders-Loehr, 1982) and on the other hand, schizokinen – as well as other siderophores – is able to complex copper (McKnight & Morel, 1979, 1980). This activity was found to be required for alleviating copper toxicity (Clarke, Stuart, & Sanders-Loehr, 1987).



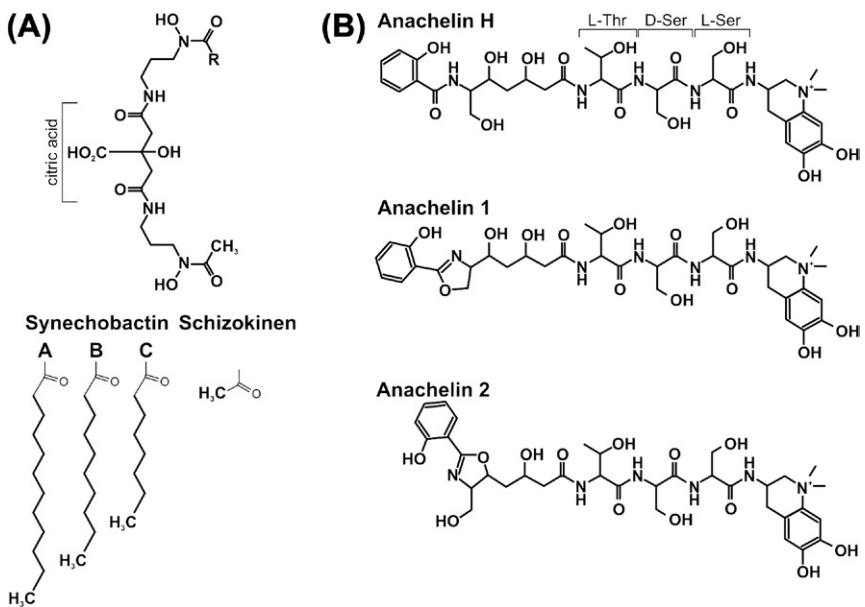
**Table 3.1** Siderophores produced by cyanobacteria

Siderophore type	Class/species	Structure	References
Hydroxamate	<i>Synechococcus</i> sp. PCC 7002	Synechobactin A-C	Armstrong & van Baalen, 1979; Ito & Butler, 2005; Wilhelm & Trick, 1994
	<i>Synechococcus</i> sp. PCC6031		Wilhelm & Trick, 1994
	<i>Synechococcus</i> sp. PCC 6908		Wilhelm & Trick, 1994
	<i>Synechococcus</i> sp. PCC 7942		Trick & Kerry, 1992; Wilhelm & Trick, 1994
	<i>Synechococcus</i> sp. WH 8101		Wilhelm & Trick, 1994
	<i>Anabaena catenula</i>		Wilhelm & Trick, 1994
	<i>O. tenuis</i>		Wilhelm & Trick, 1994
	<i>M. aeruginosa</i>		McKnight & Morel, 1979
	<i>Anacystis nidulans</i>		McKnight & Morel, 1979
	<i>Gloeocapsa alpicola</i>		McKnight & Morel, 1979
	<i>A. flos-aquae</i>		McKnight & Morel, 1979; Murphy et al., 1976
	<i>A. variabilis</i>		Trick & Kerry, 1992
	<i>Anabaena</i> sp. PCC 6411	Schizokinen	Goldman et al., 1983; Simpson & Neiland, 1976
	<i>Aanabaena</i> sp. PCC 7120	Schizokinen	Goldman et al., 1983; Lammers & Sanders-Loehr, 1982
	<i>A. cylindrica</i> Lemm 7122		Goldman et al., 1983
	<i>A. cylindrica</i> Lemm 1611		Goldman et al., 1983

Continued

**Table 3.1** Siderophores produced by cyanobacteria—cont'd

Siderophore type	Class/species	Structure	References
Catecholate	<i>O. tenuis</i>		Brown & Trick, 1992; Wilhelm & Trick, 1994
	<i>Synechococcus</i> sp. PCC6031		Wilhelm & Trick, 1994
	<i>Synechococcus</i> sp. PCC 6908		Wilhelm & Trick, 1994
	<i>Synechococcus</i> sp. WH 8101		Wilhelm & Trick, 1994
	<i>A. catenula</i>		Wilhelm & Trick, 1994
	<i>Synechococcus</i> sp. PCC 7002		Barbeau et al., 2003; Wilhelm & Trick, 1994
	<i>A. cylindrica</i>	Anachelin H, anachelin-1, -2	Beiderbeck et al., 2000; Itou et al., 2001



**Figure 3.3** Siderophore structures.

Additionally, hydroxamate-type siderophores were identified to be secreted by the coastal marine cyanobacteria *Synechococcus* sp. PCC 7002 (Armstrong & van Baalen, 1979), which were later on characterized as the amphiphilic hydroxamate-type siderophores synechobactin A–C. These are composed of a citric acid backbone, related to schizokinen, but different in the length of their fatty acid tail attached to the second  $\alpha$ -hydroxamate group (Fig. 3.3B; Ito & Butler, 2005). The fatty acid tail is thought to enhance the affinity of the siderophore for the bacterial membrane surface. Similar to many other Fe(III) chelators, synechobactin is photoreactive: the ligand undergoes a light-induced charge transfer reaction leading to oxidative cleavage resulting in a hydrophilic peptide fragment and a fatty acid tail fragment. In the process, bound Fe(III) is reduced to Fe(II) (Barbeau, Rue, Trick, Bruland, & Butler, 2003). The physiological significance of the photoreactivity of siderophores remains to be established.

Certain cyanobacteria can produce catecholate-type siderophores, similar to anachelin produced by *Anabaena cylindrica*. It coordinates iron with one catecholate and one 2-hydroxyphenyl-oxazoline system. Its structure is unusual, combining a polyketide, a central tripeptide consisting of L-Thr, D-Ser, and L-Ser and an alkaloid building block (Gademann & Bethuel, 2004a). Initially, two forms of anachelin were isolated; anachelin H, containing a terminal salicylamid and anachelin 1 containing a terminal oxazoline ring (Beiderbeck, Taraz, Budzikiewicz, & Walsby, 2000), while a subsequent study identified anachelin 1 and an isomeric form thereof (anachelin 2) instead of anachelin H (Fig. 3.3C; Itou, Okada, & Murakami, 2001). It was suggested that the only biologically active compound is anachelin H and that the oxazoline ring of anachelin 1 and 2 are formed under dehydrating conditions during purification. Anachelin H has a compact folded conformation as determined by NMR (Gademann & Bethuel, 2004a).

Siderophores have been recently introduced into biotechnology research (Gademann, Kobylinska, Wach, & Woods, 2009). For example, a synthetic anachelin chromophore was coupled to metal oxide surfaces linked via PEG to the antibiotic vancomycin producing a non-fouling antimicrobial surface. The usage of these surfaces for antimicrobial implants such as catheters and stents was suggested (Gademann et al., 2009).

## 2.2. Siderophore Synthesis

At least three different systems were reported to be involved in siderophore synthesis in cyanobacteria; two of them belonging to the non-ribosomal peptide synthetases (NRPSs), which catalyse the peptide bonds between

amino acid monomers of the siderophore backbone. The core module of an NRPS consists of three domains: the adenylation (A) domain for activation of the selected amino acid monomer, a peptidyl carrier domain (P) for transferring the monomers to various catalytic sites and a condensation (C) domain for forming peptide bonds between the monomers (Du & Shen, 2001; Marahiel, Stachelhaus, & Mootz, 1997). In several cases, NRPSs are accompanied by polyketide synthases (PKSs) that are known to catalyse the condensation of carboxylate groups (Staunton & Weissman, 2001). The two main classes differ in the number of protein units; while type I PKSs are multifunctional enzymes, type II PKSs are made of separate enzymes with sole functions (Shen, 2003).

The third pathway for siderophore synthesis is NRPS-independant (NIS), as identified for the synthesis of aerobactin in *Escherichia coli* and rhizobactin 1021 in *Sinorhizobium meliloti* (Challis, 2005). Thus, while the other two synthesis pathways are important for hydroxamate and catecholate-type siderophores, NIS has only been demonstrated for hydroxamate-type siderophores. For aerobactin, the two synthetases IucD and IucB are known to catalyse the synthesis of the terminal hydroxamate group important for iron coordination. The enzymes IucA and IucC are responsible for amid bond formation that links the carboxylate and diamine units together. The four synthetases RhbC, RhbD, RhbE and RhbF, participating in the rhizobactin synthesis, show similarities with the IucA/B/C/D enzymes. Additionally, the two PLP-dependant-like enzymes RhbA and RhbB are supposed to catalyse 1,3-diaminopropane before the hydroxamate group is formed (Challis, 2005). Several genes coding for NRPSs and PKSs are annotated in the genomes of cyanobacteria (Silva-Stenico *et al.*, 2011), however, the siderophores produced by these genes could not be identified.

Components of the NIS pathway were identified by a bioinformatic survey only in the freshwater cyanobacteria *Anabaena* sp. PCC 7120, *Anabaena variabilis* ATC 29413, in the marine cyanobacteria *Synechococcus* sp. PCC 7002, *Synechococcus* sp. and *Prochlorococcus* sp. while in the same study, NRPS components were identified in more than 50% of the analysed species (Hopkinson & Morel, 2009). Whether the absence of NIS or NRPS components in the genome of the other species indeed indicate absence of siderophore synthesis machineries or simply a false discovery rate of the survey remains to be analysed.

The only gene cluster which was further investigated, consists of the genes spanning *all2658* to *all2635* (in *Anabaena* sp. PCC 7120; Jeanjean *et al.*, 2008) including seven NRPSs and two PKSs (Kaneko *et al.*, 2001).

A mutant lacking the whole gene cluster showed a growth defect under iron-limiting conditions and produced only a low level of siderophores suggesting an importance of this cluster for siderophore production. However, the viability of the mutant as well as the remaining secretion of siderophores point to the presence of additional cluster(s) involved in siderophore synthesis in this organism (Jeanjean et al., 2008). Whether the enzymes encoded by all2658–all2635 are responsible for the synthesis of several or just one siderophore could not be determined. The cluster was upregulated under iron starvation, oxidative stress and copper limitation (Jeanjean et al., 2008).

The genes of a second cluster described in *Anabaena* sp. PCC 7120 show similarities with the *rhb* genes coding for the NIS pathway of rhizobactin (Nicolaisen et al., 2008). As rhizobactin is a citrate-based siderophore, a possible role of the encoded enzymes in the synthesis of schizokinen is suggested. As mentioned previously, genes encoding for the NIS pathway are not widespread among cyanobacteria. Of the analysed bacteria, only *Synechococcus* sp. PCC 7002 and *A. variabilis* contain putative genes encoding for this biosynthesis pathway. Both organisms are known to produce hydroxamate-type siderophores (Hopkinson & Morel, 2009; Ito & Butler, 2005; Trick & Kerry, 1992).

The biosynthesis pathway of the more complex siderophore anachelin is not solved yet although it could be synthesized synthetically (Gademann & Bethuel, 2004b). Anachelin is considered as a natural hybrid containing fragments of different biosynthetic origin, a salicylate unit, a mixed polyketide/amino acid fragment, a tripeptide part and an unusual alkaloid fragment. The salicylate could emanate out of a PKS-dependant pathway (Gademann & Portmann, 2008).

### 2.3. Siderophore Export and Uptake Cycles

Siderophore secretion by cyanobacteria is an intriguing process. The only cyanobacterial siderophore secretion pathway studied in detail, so far, is in *Anabaena* sp. PCC 7120. Export through the inner membrane is suggested to occur via transporters of the resistance, nodulation and cell division (RND), the major facilitator (MFS) or the ATP-binding cassette (ABC) superfamilies. Transport through the outer membrane is mediated by a TolC-type protein (Bleuel et al., 2005; Miethke & Marahiel, 2007). In *Anabaena* sp. PCC 7120, the MFS-type plasma membrane protein of SchE was shown to be involved in the export of schizokinen. A deletion mutant of *schE* showed a drastic reduction in secretion (Nicolaisen et al., 2010). The TolC-like protein of *Anabaena* sp. PCC 7120, HgdD, seems to be involved in secretion of siderophores through the outer membrane (Fig. 3.4A; Nicolaisen et al., 2010).

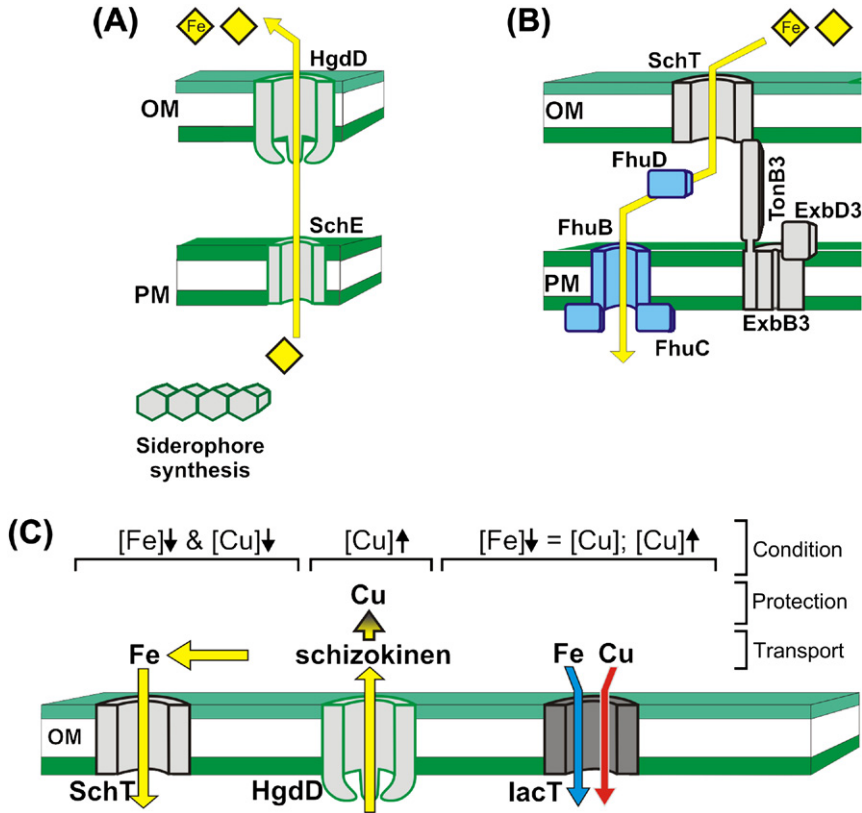
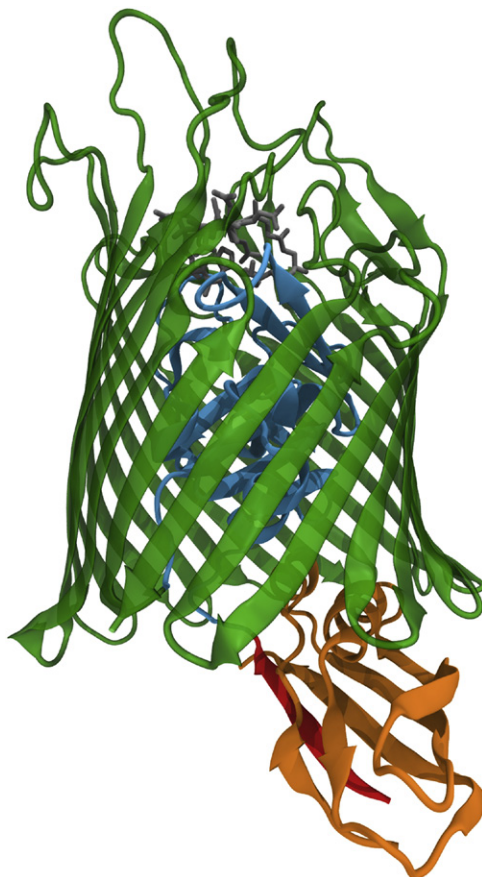


Figure 3.4 Iron uptake pathway in *Anabaena* sp. PCC 7120. See the colour plate.

Siderophore uptake was extensively studied in *E. coli* and *Pseudomonas aeruginosa* (Faraldo-Gómez & Sansom, 2003). It was demonstrated that siderophores bind with high affinity to specific transporters in the outer membrane of Gram-negative bacteria (TonB-dependant transporters, TBDTs). Despite the substrate diversity of TBDTs, the structure of these transporters seems to be similar consisting of a 22-stranded  $\beta$ -barrel embedded in the membrane and an N-terminal plug domain that is located within the barrel (Fig. 3.5). Among TBDT sequences of different species, the majority of identity is in the plug domain, whereas the  $\beta$ -barrel is poorly conserved in sequence. TBDT substrates bind, primarily, to the extracellular pocket of the transporter consisting of substrate-binding sites, composed of the plug and the barrel domain. In most cases, these substrate-binding sites lack sequence conservation (Chimento, Kadner, & Wiener, 2005) indicating the specificity of the transporters for different substrates. These include, beside



**Figure 3.5** Structure of a TBDT. See the colour plate.

siderophores, haeme, vitamin B12 and several other organic and inorganic compounds (Noinaj, Guillier, Barnard, & Buchanan, 2010; Schauer, Rodionov, & de Reuse, 2008).

According to bioinformatic genome analyses, various genes coding for putative TonB-dependant receptors can be found in several cyanobacterial classes and species ranging from 1 to 33 transporters present in one organism (Mirus, Strauss, Nicolaisen, von Haeseler, & Schleiff, 2009). It was suggested that the number of TBDTs is dependant on the ecological niche in which the organism thrives (Hopkinson & Morel, 2009; Mirus et al., 2009; Stevanovic, Hahn, Nicolaisen, Mirus, & Schleiff, 2012).

In the genome of *Anabaena* sp. PCC 7120, 22 different genes code for putative TBDTs can be identified by bioinformatical means. Not all of these

outer membrane proteins bind to typical siderophores. Two are homologues of the vitamin B12 transporter BtuB characterized in *E. coli*, another two are homologues of the haeme transporter HutA characterized in *Vibrio cholera* (Mirus *et al.*, 2009). Haeme and Vitamin B12 belong to the group of porphyrins that are able to chelate iron or cobalt, respectively. Interestingly, one of the BtuB and one of the HutA-type transporters are under the control of FurB (Napolitano *et al.*, 2012), which might hint at a different metal specificity.

To date, only one cyanobacterial TonB-dependant receptor is known to transport a particular siderophore. This schizokinen transporter, SchT, is encoded by *alr0379* in *Anabaena* sp. PCC 7120 (Nicolaisen *et al.*, 2008). It is homologous to the hydroxyl-carboxylate siderophore transporter IutA, characterized as the ferric aerobactin transporter in *E. coli* (Mirus *et al.*, 2009). While the mutation in *schT* drastically reduces the uptake of schizokinen (Nicolaisen *et al.*, 2008), two additional transporters of the IutA type are encoded in the genome (*alr2209* and *alr2581*; Mirus *et al.*, 2009). The function of these two genes has to be explored.

It can be proposed that several of the identified FhuA, ViuA, HutA and BtuB-type TBDTs of *Anabaena* sp. PCC 7120 are involved in transport of siderophores as this cyanobacterium contains multiple gene clusters encoding for proteins involved in siderophore synthesis (Jeanjean *et al.*, 2008; Nicolaisen *et al.*, 2008) and a second siderophore of a yet unknown nature (Nicolaisen *et al.*, 2010). Furthermore, it has been shown that cyanobacteria are capable of taking up siderophores from different bacterial domains and even from fungi. For example, *Anabaena* sp. PCC 7120 and *Synechocystis* sp. PCC 6803 can utilize ferric aerobactin originating from *E. coli* (Goldman *et al.*, 1983; Kranzler *et al.*, 2011). In addition, the differences observed in the expression pattern of the genes encoding for the 22 TBDTs present in *Anabaena* sp. PCC 7120 under iron-, copper- and nitrogen-limiting conditions (Mirus *et al.*, 2009) and the dependence of other bacterial TBDTs on metals such as nickel and cobalt (Schauer *et al.*, 2008) allows us to hypothesize on additional metal-dependant iron-siderophore uptake systems able to take up siderophores complexed to metals other than iron.

In *Synechocystis* sp. PCC 6803, the four genes *sll1206* (IutA-type), *sll1406*, *sll1409* and *slr1490* (all FhuA-type), coding for putative siderophore transporters in the outer membrane, were investigated. Mutants thereof did not show a growth phenotype in Fe-depleted media. Furthermore, no reduction in Fe(II) or Fe(III) uptake activity was observed (Katoh, Hagino,



Grossman, & Ogawa, 2001). However, these results do not exclude the possible involvement of these as well as other putative transporters in iron uptake under different growth conditions.

## 2.4. Siderophore Transport across the Periplasm and the Plasma Membrane

Energy for the active transport of the ferric iron–siderophore complex is supplied by a ‘Ton’ system in the periplasmic membrane. It consists of the energy transducing unit, TonB, anchored to the periplasmic membrane (Wang & Newton, 1971) and two stabilizing units: the integral membrane proteins, ExbB and ExbD, also anchored to the periplasmic membrane. The interaction between TBDT and the Ton system is accomplished by TonB. The TBDT contains a TonB–box positioned in front of its N-terminal plug domain, which is recognized by TonB (Fig. 3.5). Siderophore transport is, at first, blocked by the plug domain that prevents passage of the siderophore through the TBDT channel. The interaction between TonB and the siderophore-loaded TBDT results in conformational changes in the plug domain, which induces the transfer of siderophores through the pore of the TBDT. TonB functions as an energy transducer by coupling the proton-motive force of the cytoplasmic membrane to the outer membrane transporter (e.g. Faraldo-Gómez & Sansom, 2003).

Knowledge about the Exb/TonB system and particularly about its function in siderophore uptake in cyanobacteria is rather sparse. Four putative *tonB* genes and three *exbB/D* gene cluster were identified in the genome of *Anabaena* sp. PCC 7120 (Stevanovic et al., 2012). Thus, the complexity of these systems is lower than that found for TBDTs (Stevanovic et al., 2012). Expression profiling and mutant analysis in *Anabaena* sp. PCC 7120 revealed a possible role of *tonB3* and the *exbB3/D3* gene cluster in siderophore-dependant iron uptake (Fig. 3.4B). These genes were upregulated under iron-limiting conditions and the mutants displayed an enhanced expression of genes coding for siderophore synthesis and secretion. Additionally, the transcript levels of the *tonB3* and *exbB3/D3* genes are higher in mutants with reduced siderophore cycling capacity (Stevanovic et al., 2012).

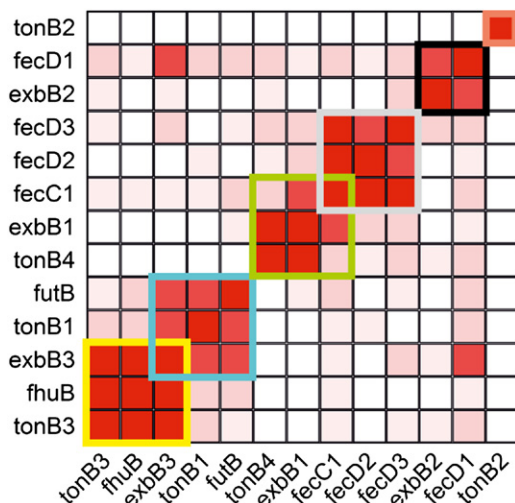
The *exbB2* gene, which is not found in the genomic cluster together with a gene coding for ExbD2, is only expressed under nitrogen starvation, while *exbB1/exbD1* expression seems to sense the ratio between iron and other metals. Among the other three *tonB* genes the transcription of one is strongly enhanced when iron is present at semi-toxic concentrations, while

for the other two a relation to copper was described as well. However, these TonB proteins do not contain the histidine in the first transmembrane domain which is thought to be essential for the function of the TonB in *E. coli* (Larsen *et al.*, 2007). Thus, their relation to iron transport remains to be established.

In general, transport of iron-loaded siderophores across the periplasm and the inner membrane is mediated by a three-component system. It consists of a periplasmic binding protein (e.g. FhuD/FutA/FecA), a membrane-embedded permease (e.g. FhuB/FutB/FecCD) and an ATP-binding protein (e.g. FhuC/FutC/FecB). The FhuD/B/C-system, responsible for the transport of ferrichrome, is well characterized in *E. coli* (Krewulak & Vogel, 2011).

In *Anabaena* sp. PCC 7120, five gene clusters with similarities to the Fut-, Fec- and Fhu-system are annotated. Out of these, only one cluster seems to be related to periplasmic iron-siderophore uptake, namely the fhu-cluster (Fig. 3.4B). The genes present in the cluster are differentially regulated. It was reported that the expression of the *fhu* genes is influenced by ExbB3/D3 and TonB3. This notion is based, for example, on the observation that the *fhu* genes were not expressed in the *exbB3*- and *tonB3*-deficient mutants. Additionally, transcript levels were reduced in mutants of siderophore secretion and uptake and the *fhuB* mutant showed an iron starvation phenotype.

While for the two putative fec-gene clusters, no correlation between expression and presence of iron could be observed, their expression were found to differ under deviant copper (high copper for gene cluster 1 and low copper for gene cluster 2) and nitrogen-limiting conditions. Out of the fut-gene cluster, only *futB* showed altered expression under elevated copper concentrations (Stevanovic *et al.*, 2012). The correlation of the expression profiles for the different components is shown in Fig. 3.6. Remarkably, besides genes expressed under iron-limiting conditions (Fig. 3.6, yellow frame), we found at least five other clusters. The *futB*, *tonB1* and *exbB3* are also induced under high copper conditions (blue frame), while *fecC1*, *tonB4*, and *exbB1* are induced under high iron and copper (green frame). Strikingly, the transport components *fecC1*, *fecD2* and *fecD3* are induced under high iron and low copper concentrations (grey frame), and *fecD1* and *exbB2* under limiting nitrogen (black frame). This type of regulation appears to be unique and at this stage, it is unknown which function this regulation has.



**Figure 3.6** The correlation of the expression profiles for the different iron transport components. See the colour plate.

## 2.5. The Relation between Siderophore Iron Uptake and Copper Uptake

The analysis of iron uptake by *Anabaena* sp. PCC 7120 revealed a relation between iron and copper uptake. The gene product of *all4026* annotated as iron and copper transporter IacT was the only TBDT found to be homologous to a catecholate-type transporter (ViuA from *V. cholera*; [Mirus et al., 2009](#)). The transported siderophore, if existing, could not be defined. Nevertheless, an enhanced secretion of siderophores in the *iacT* mutant was observed ([Nicolaisen et al., 2010](#)). In addition, the mutant shows reduced citrate-based iron transport and reduced iron and copper levels when compared to wild type ([Nicolaisen et al., 2010](#)). Accordingly, transport of both iron and copper was reduced in the mutant. It has been suggested that IacT is able to transport iron and copper ([Nicolaisen et al., 2010](#)). On the basis of these observations, a crosstalk between iron and copper was proposed.

SchT transports the siderophore schizokinen in its ferrated form. However, schizokinen is able to complex copper, which is not transported across the membrane ([Clarke et al., 1987](#)). The latter can be explained as the ferric siderophore complex is known to be octahedral, whereas the cupric siderophore complex is square planar ([Clarke et al., 1987](#)). Thus, in situations where the copper concentration exceeds the iron concentration, the majority of schizokinen will be complexed with copper leading to iron starvation.

As a consequence of the function of schizokinen in copper detoxification under high copper concentrations, an alternative transport system is required. A system for cotransporting iron and copper would be of evolutionary advantage. It would only be active when copper concentrations are high and would ensure iron and copper uptake in situations where copper is largely stored in siderophore complexes. Accordingly, iron uptake by *Anabaena* sp. PCC 7120 grown in the absence of iron but in the presence of copper is lower when compared to cells grown in the absence of both metals (Nicolaisen *et al.*, 2010) which supports copper uptake under iron-limiting conditions (Fig. 3.4C). IacT was proposed as the outer membrane protein involved in this process. In line, in the *iacT* overexpression mutant, two genes (all7619 and all7632) coding for homologous to CusB involved in copper homeostasis in *E. coli* (Rensing & Grass, 2003) showed reduced expression and the copper content was increased as compared to wild type (Nicolaisen *et al.*, 2010).

Such a model is consistent with the observed differential expression of the energizing and transport system. *TonB1* and *tonB4* as well as *fecC1* and *fecD1* are expressed under high copper concentrations (Stevanovic *et al.*, 2012). Furthermore, FutA2 in *Synechocystis* sp. PCC 6803 known to bind to Fe(III) *in vitro* could be linked to copper binding *in vivo* (Badarau *et al.*, 2008). The *futA2* mutant accumulated iron and copper in the periplasm, but no longer in plastocyanin (the major copper protein in the cells) leading to the suggestion that FutA2 is involved in copper homeostasis in addition to its function of iron transport (Waldron *et al.*, 2007). The relationship between IacT and the periplasmic components is not yet established, although, additionally to this TBDT, an involvement of a periplasmic binding protein in the uptake of both iron and copper was described (Stevanovic *et al.*, 2012).

## 2.6. Reductive Iron Uptake

### 2.6.1. Non-siderophore-mediated Fe uptake

Siderophore-mediated iron uptake has been studied extensively in Gram-negative bacteria (Braun & Killmann, 1999; Braun & Hantke, 2011; Sandy & Butler, 2009). Similarly, most studies on iron acquisition in cyanobacteria, also Gram-negative prokaryotes, focused on siderophore biosynthesis and uptake, as detailed above. However, siderophore-mediated iron uptake cannot account for iron uptake in all cyanobacteria. Bioinformatic analysis of two molecular systems implicated in siderophore biosynthesis, NRPSs and modular PKSs, was performed on both freshwater and marine cyanobacterial genomes (Ehrenreich, Waterbury, & Webb, 2005). While both NRPS and PKS genes were prevalent among filamentous and heterocystous strains, they were

notably absent from many *Synechocystis*, *Prochlorococcus* and *Synechococcus* species. Further bioinformatic analysis was done by [Hopkinson and Morel \(2009\)](#) including the prevalence of NIS pathways for siderophore biosynthesis as well as TonB-dependent transporters in cyanobacteria. Neither of these pathways was found in marine unicellular picocyanobacteria ([Hopkinson & Morel, 2009](#)). Several strains that have no known siderophore biosynthesis genes do have TonB-dependent transporters encoded in their genomes. The *Synechocystis* PCC 6803 genome contains four putative outer membrane siderophore transporters (Kato, 2001). Some ferrisiderophore complexes are bioavailable to this organism but no siderophore secretion was detected in CAS assays ([Kranzler et al., 2011](#)).

An alternative Fe uptake strategy characterized in many plants, yeast and eukaryotic algae is the extracellular reduction of Fe(III) or Fe(III) chelates by a reductase on the plasma membrane and subsequent uptake of Fe' ([Allen, del Campo, Kropat, & Merchant, 2007](#); [Kustka, Allen, & Morel, 2007](#); [Lesuisse & Labbe, 1989](#); [Morrissey & Guerinot, 2009](#); [Schmidt, 1999](#); [Shaked, Kustka, & Morel, 2005](#)). Currently, there is some evidence for extracellular reduction of Fe(III) species before transport in a number of non-photosynthetic prokaryotes ([Schröder, Johnson, & De Vries, 2003](#); [Vartivarian & Cowart, 1999](#)).

### **2.6.2. Uptake of free, unchelated, inorganic iron**

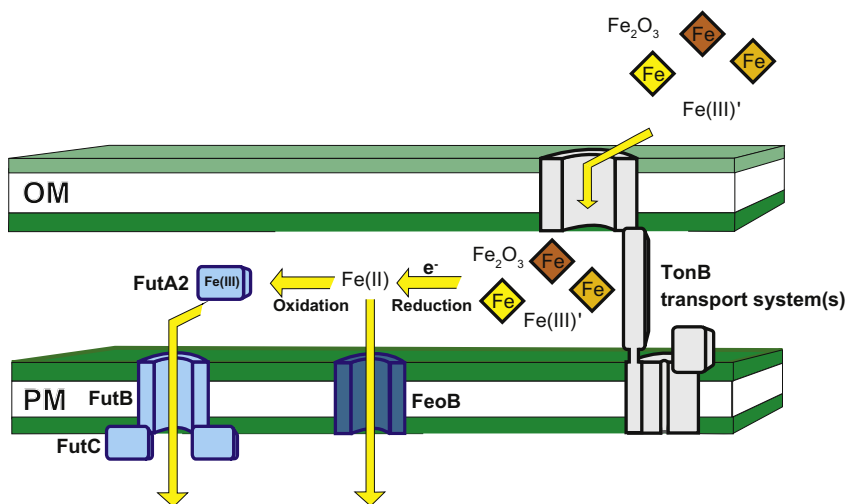
The existence of an iron uptake pathway for cyanobacteria that involves a reductive step has been targeted in a few studies ([Kranzler et al., 2011](#); [Lis & Shaked, 2009](#); [Rose et al., 2005](#)). [Rose et al. \(2005\)](#) demonstrated that superoxide-mediated extracellular iron reduction enhances Fe uptake in the marine cyanobacterium *Lyngbya majuscula*. Using a chemiluminescence system, extracellular Fe(II) production by *L. majuscula* was demonstrated. Reduction of iron by superoxide increases iron availability in this organism and superoxide production seems to be metabolically related. When iron was applied in the form of FeEDTA, the addition of superoxide dismutase inhibited Fe uptake by 94% suggesting that superoxide is involved in the iron uptake pathway. However, the application of the Fe(II)-specific ligand, ferrozine (FZ), did not inhibit Fe uptake suggesting that organically complexed ferrous iron may be directly transported ([Rose et al., 2005](#)).

Both natural *Synechococcus* populations in the Gulf of Aqaba as well as axenic *Synechococcus* WH8102 cultures may also employ reductive Fe uptake in order to acquire organically bound iron ([Lis & Shaked, 2009](#)). Both were able to acquire iron bound to the strong Fe(III) chelator, ferroxamine

B (DFB), but were inhibited by the application of excess chelator. This may suggest that organic iron is acquired in these *Synechococcus* sp. by means of an extracellular reductive step, in which the ferric DFB is reduced, Fe(II) released and Fe' is transported by the cell (Lis & Shaked, 2009). This possibility is consistent with the Fe(II)s model developed for eukaryotic phytoplankton, which predicts that inhibition of Fe uptake by excess ligand results from competition between the cell and ligand for free Fe(II) formed at the cell surface (Shaked *et al.*, 2005).

Recently, a reductive iron uptake strategy was demonstrated for *Synechocystis* PCC 6803 (Kranzler *et al.*, 2011). As previously mentioned, its genome contains no known siderophore biosynthesis genes (Ehrenreich *et al.*, 2005) although Fe(II) and Fe(III) transporters in the inner membrane were identified: FutABC and FeoB were identified by Katoh, Hagino, Grossman *et al.* (2001) as Fe(III) and Fe(II) transporters, respectively. Recently, Jiang, Lou, Du, Price, and Qiu (2012) identified a CDF-type transporter which may be involved in Fe(III) transport. In order to probe the existence of a reductive uptake strategy, short-term Fe transport assays were conducted in the presence of the membrane impermeable, Fe(II)-specific ligand, ferrozine (Fig. 3.1B). When both Fe' and ferrisiderophore complexes were applied as substrates, FZ inhibited iron uptake suggesting that free Fe(II) is formed before transport (Kranzler *et al.*, 2011). Fe' transport rates revealed a high-affinity uptake system under Fe-limited conditions with an environmentally relevant  $K_m$  in the sub-nanomolar range (Kranzler *et al.*, 2011).

In Gram-negative prokaryotes, iron reduction may occur outside of the cell, on the surface of the outer membrane or in the periplasmic space. Iron reduction in the bulk medium cannot contribute significantly towards uptake, given the short residence time of Fe(II) in an oxic environment. Therefore, biologically relevant iron reduction must take place either on the surface of the outer membrane or in the periplasm. While Fe transport processes through the outer membrane can be energetically coupled (Braun & Endriss, 2007; Mirus *et al.*, 2009), there is no evidence for the presence of redox processes in this membrane. Redox reactions do take place in the periplasm of *Synechocystis* PCC 6803, as evidenced by the ability to form and break disulfide bonds for example (Pils & Schmetterer, 2001; Rukhman *et al.*, 2005; Singh *et al.*, 2008). However, the Fe(II) trapping agent used to assay for reduction (FZ) does not cross the plasma membrane (Garg, Rose, Godrant, & Waite, 2007; Kustka, Shaked, Milligan, King, & Morel, 2005; Shaked *et al.*, 2005). Its ability to cross the outer membrane of a cyanobacterial cell is unknown. Fe(II)–FZ<sub>3</sub> complexes are not available for



**Figure 3.7** Uptake pathway model for *Synechocystis* PCC 6803. See the colour plate.

uptake (Rose et al., 2005; Shaked, Kustka, Morel, & Erel, 2004). Therefore, it is possible that in *Synechocystis* PCC 6803, reduction occurs in the periplasmic space before transport through the plasma membrane (Fig. 3.7). In other cyanobacterial species, some work has been done to demonstrate the bioavailability of Fe'. In the siderophore-producing cyanobacterium *Anabaena flos-aquae*, siderophore-independent uptake of inorganic iron was shown under Fe limitation (Wirtz, Treble, & Weger, 2010). The relations between siderophore and non-siderophore uptake pathways in cyanobacteria warrant further investigation.

### 2.6.3. Reductive siderophore uptake

Ferrisiderophore reduction outside the plasma membrane has been studied in the nonphotosynthetic bacterium *P. aeruginosa*. In this organism, siderophores are secreted by the cell, ferrisiderophores are actively transported through the outer membrane and then the complex is reduced in the periplasm before transport across the plasma membrane. The siderophores are recycled from the periplasm back into the environment (Greenwald et al., 2007). In *Synechocystis* PCC 6803, short-term iron uptake of Fe' was compared with that of FeDFB. DFB binds Fe so strongly (Martell and Smith, 1975) that with the excess chelator used, no Fe' was present in the medium (Kranzler et al., 2011). Experiments were done using medium containing either 0.12 nM Fe' or 100 nM FeDFB. Despite the large difference in the pool of bioavailable iron, Fe' was transported with remarkable efficiency

and supported uptake rates similar to those measured for FeDFB. This finding is consistent with data from work with eukaryotic algae demonstrating that free iron is the most biologically available form for uptake (Hudson & Morel, 1989; Morel *et al.*, 2008) and that Fe' uptake rates are 100–200 times faster than uptake of FeDFB (Maldonado & Price, 2001; Shaked *et al.*, 2005). Nevertheless, the data also exhibited considerable rates of FeDFB and Feaerobactin transport. Ferrated siderophore uptake was inhibited by FZ. These results suggested that the reductive uptake system identified in *Synechocystis* PCC 6803 is able to utilize organically bound Fe of an exogenous source.

## 2.7. Fe' Transport through the Plasma Membrane

The reduction of a variety of inorganic and organic iron sources, before transport through the plasma membrane, eliminates the need for specific ferrisiderophore transporters. For internalization of Fe, this type of mechanism requires only free iron transporters in the plasma membrane. Following reduction, iron may be transported as Fe(II) or, in some cases, reoxidized to Fe(III) and then transported (Askwith *et al.*, 1994; Maldonado *et al.*, 2006; Stearman, Yuan, Yamaguchi-Iwai, Klausner, & Dancis, 1996). These possibilities are consistent with two of the transport systems identified in the plasma membrane of *Synechocystis* PCC 6803: FutABC and FeoB, suggested to transport free Fe(III) and Fe(II), respectively (Badarau *et al.*, 2008; Katoh, Hagino, Grossman *et al.* 2001; Katoh, Hagino, & Ogawa, 2001). Disruption mutants in the *fut* and *feoB* genes are viable; however, a homo-plasmic mutant in which both transport systems are inactivated could not be segregated (Katoh, Hagino, & Ogawa, 2001) suggesting that these two systems are responsible for most of the iron accumulation in this organism.

While the reduction of organically bound iron results in its release from the complex, thus enabling uptake, reduction of Fe' may seem redundant in the presence of both Fe(II) and Fe(III) transporters. Nonetheless, this strategy is well documented in many eukaryotic microorganisms such as *Saccharomyces cerevisiae* (Stearman *et al.*, 1996), the centric diatoms *Thalassiosira weissflogii* and *Thalassiosira pseudonana* (Shaked *et al.*, 2005), and the green algae *Chlamydomonas reinhardtii* (La Fontaine *et al.*, 2002).

## 2.8. Environmental Implications

Thus far, two iron uptake strategies have been demonstrated for cyanobacteria: siderophore-mediated transport and reductive iron uptake. Given its broad range of iron substrates found within natural aquatic environments,



reductive iron uptake may be applied to a variety of Fe sources. This generalist-type strategy would confer an obvious advantage to organisms living in a dilute and heterogeneous aquatic environment, in which iron is bound by many different organic chelators, siderophores included (Hopkinson & Morel, 2009).

This was demonstrated in a numerical model that quantified the effectiveness of endogenous siderophore-mediated uptake and reductive Fe uptake (Völker & Wolf-Gladrow, 1999). High-affinity transport of specific ferrisiderophore complexes is effective in densely populated environments where cell numbers offset diffusive losses. In a dilute aqueous environment where diffusive losses are imminent, endogenous siderophore uptake is inefficient. In this type of environment, reductive Fe uptake is an effective strategy in the acquisition of organically bound iron. It is important to note that these two transport systems can co-exist in one organism.

A recent study of iron transporters in marine prokaryote genomes and metagenomes identified uptake mechanisms for Fe(III), Fe(II) and organically bound iron. Bioinformatic analysis of environmentally abundant microbes was used to identify iron uptake strategies prevalent in the marine environment. Fe(III) ABC transporters were most commonly found among marine bacteria. Among most known free-living bacteria such as picocyanobacteria, TonB-dependent transporters were absent (Hopkinson and Barbeau, 2012). The authors also tested for statistical associations between Fe transport genes and found a negative correlation between ABC transport systems and FeoB transporters (Hopkinson and Barbeau, 2011).



### 3. INTRACELLULAR FE HOMEOSTASIS

Once transported into the cell, iron can be utilized for the assembly of active cofactors such as haeme, cytochromes or Fe–S clusters. A major factor in the production of Fe–S cluster assembly is the SufBCDE system. This system is regulated by the iron status of the cell but also by other environmental inputs such as light intensity (Balasubramanian, Shen, Bryant, & Golbeck, 2006; Seki et al., 2006). Additionally, the involvement of monothiol Grx proteins as Fe–S clusters donors was suggested (Picciocchi, Saguez, Boussac, Cassier-Chauvat, & Chauvat, 2007). Cytochrome assembly takes place by a number of routes and exhibits strong divergence between different organisms (review by Kranz et al., 2002).

Alternatively, iron can be stored in ferritin complexes, which form multimeric spherical complexes inside which ferric oxide is deposited

(Le Brun, Crow, Murphy, Mauk, & Moore, 2010). The deposition of iron is accompanied by deposition of phosphate. In cyanobacteria, three different types of ferritin family proteins were identified. Ferritins and bacterioferritins function as ferroxidases, oxidizing Fe(II) to Fe(III), while generating hydrogen peroxide. This ferroxidase activity is carried out by the di-iron centre. Fe(III) is stored as iron oxide in the cavity at the centre of their 24-mer ultrastructure (Le Brun *et al.*, 2010).

Bacterioferritins differ from ferritins in the presence of a haeme molecule, anchored at the interface between two adjacent subunits. The role of this haeme remains poorly defined but it was suggested that it contributes to iron reduction and release from the core of the complex (Carrondo, 2003). DNA protection during starvation (DPS) family (DNA-binding proteins from starved cells) proteins have a very similar function. However, the electron acceptor in the case of DPS proteins is hydrogen peroxide instead of water.

The ferroxidase di-iron centre of DPS proteins is located between two helices. In addition, the structure of DPS proteins is shorter by one  $\alpha$ -helix, as compared to ferritins and bacterioferritins. As a result, DPS multimeric structures are 12-mer, whereas ferritins and bacterioferritins form larger 24-mer structures. From an evolutionary standpoint, DPS proteins represent a more diverse group than the other ferritin families with members functioning as iron storage proteins, DNA-binding proteins protecting against oxidative stress, cold shock proteins, neutrophile activators or pili components (Zeth, Offermann, Essen, & Oesterhelt, 2004). These activates are not necessarily connected to the ferroxidase activity.

To date, functional analysis of the role of these proteins in cyanobacteria is largely limited to freshwater species. In *Synechocystis* PCC 6803, it was found that *bfr* genes belong to a subfamily of bacterioferritin genes, in which one gene codes for a protein with a conserved haeme ligand and the other codes for a protein with conserved di-iron centre ligands (Bertani, Huang, Weir, & Kirschvink, 1997). Inactivation mutants lacking either of the two proteins exhibited a loss of ~50% of the cellular iron quota and the induction of the iron stress response pathway even under iron-replete growth conditions (Keren, Aurora, & Pakrasi, 2004). The double insertion mutant did not display a more severe phenotype, indicating that both proteins are required for effective iron storage. Isolated bacterioferritins from *Synechocystis* PCC 6803 contain 2300 atoms of iron and 1500 molecules of phosphate per complex (Laulhere & Briat, 1993).

A study of the single DPS protein of *Synechocystis* PCC 6803 (MrgA) demonstrated a reduction in the ability of  $\Delta mrgA$  cells to withstand exposure to hydrogen peroxide (Li, Singh, McIntyre, & Sherman, 2004). In addition, MrgA was found to have an important role in iron homeostasis.  $\Delta mrgA$  cells grew much slower than wild-type cells when transferred from iron-sufficient to iron-deficient conditions, such as the BFR mutants (Shcolnick et al., 2007). However, unlike the BFR mutants, the internal iron quota of  $\Delta mrgA$  cells grown on sufficient iron was similar to that of wild-type cells (Shcolnick et al., 2007). An examination of an array of mutants in both bacterioferritin and DPS genes demonstrates a connection between internal iron quota, the presence of the two storage complexes and the sensitivity to externally applied oxidative stress (Scholnick et al., 2009). Bacterioferritins function as the main storage complexes, whereas DPS-type proteins function downstream in the iron storage pathway, releasing iron from bacterioferritins and transporting it towards its final destination. The combined action of the two complexes allows safe accumulation and release of iron from storage by minimizing damage resulting from interactions between free iron and the oxygen radicals that are produced in abundance by the photosynthetic apparatus (Scholnick et al., 2009).

Peña and Bullerjahn (1995) and Peña, Burkhart, and Bullerjahn (1995) isolated and studied the DPS protein of *Synechococcus* sp. PCC 7942. The genome of this organism codes for one bacterioferritin and one DPS protein. The DPS protein forms large complexes (approximately 150 kDa) and possesses both ferroxidase and catalase activity. The authors also report on binding to chromosomal DNA, which may contribute to local protection against oxidative damage. DNA binding was also reported by Franceschini, Ceci, Alaleona, Chiancone, and Ilari (2006) for the DPS protein from *Thermosynechococcus elongatus*. Interestingly, the *Synechococcus* DPS exhibits a high degree of homology to bacterioferritins and, indeed, binds haeme (Peña & Bullerjahn, 1995). The transcription of this protein is induced by iron limitation (Sen, Dwivedi, Rice, & Bullerjahn, 2000). A very similar protein was isolated from *Trichodesmium erythraeum* (Castruita et al., 2006). However, haeme binding was not reported for this protein. The transcript coding for this protein was found to be induced under nitrogen limitation, which should not be surprising when the iron requirement of the nitrogenase is considered (Sandh et al., 2011). In the *furA* overexpressing mutant of *Anabaena* sp. PCC 7120, the expression of *dpsA* was enhanced, suggesting regulation by the Fur system (Hernández, Pellicer, Huang, Peleato, & Fillat, 2007). This protein

cannot bind to DNA directly and it was suggested that its function in Fe scavenging may provide protection to the cells. As suggested for *Synechocystis* sp. PCC 6803 (Wei, Mingjia, Xiufeng, Yang, & Qingyu, 2007).

These observations point to a fundamental difficulty in annotating these proteins. On the basis of sequence data alone, it is hard to determine which group of ferritin protein it belongs to. Proximity matrix analysis of multiple sequence alignment of ferritin-coding genes from multiple cyanobacterial strains, for which complete genome sequences are available, indicates a continuum of protein sequences rather than a clear distinction into three classes. However, it is important to note that in all the fully sequenced genomes, represented in Cyanobase, ferritin homologues exist. *Prochlorococcus marinus* MED4 strain with the severely reduced genome size contains one ferritin homologue. Many of the strains contain multiple ferritin sequences. The *Nostoc punctiforme* ATCC 29133 genome, for example, contains five. Considering the diverse functions associated to members of the ferritin family, their presence as gene families should not be surprising.



## 4. IRON-DEPENDANT GENE REGULATION AND PHYSIOLOGICAL RESPONSES

The scope of physiological responses to iron limitation is vast. Studies of iron starvation have uncovered responses ranging from transcriptional regulation to changes in the quaternary structure of pigment–protein complexes. The regulation at the transcriptional level involves several layers of signal transduction. The major regulatory system is composed of Fur-type regulators and antisense RNA. However, as iron starvation is linked to many other cellular effects, the regulatory systems have a certain overlap to other stress response pathways. For example, iron limitation causes the accumulation of reactive oxygen species (ROS) by about 10-fold in comparison to normal growth as shown in *Anabaena* sp. PCC 7120 and *Synechocystis* sp. PCC 6803 (Latifi, Jeanjean, Lemeille, Havaux, & Zhang, 2005). Thus, separating the responses to iron limitation and oxidative stress is difficult.

### 4.1. Regulation of Cyanobacterial Gene Expression by Iron

Fur-mediated regulation was first identified using an *in vitro* coupled transcription translation system by which a function of Fur as Fe(II)-dependant repressor was established (Bagg & Neilands, 1987). As a consequence of and a prerequisite for the sensing of intracellular iron concentration, Fur proteins have an affinity for ferrous iron in the range of free cytosolic iron

(Keyer & Imlay, 1996). Later on, binding of other metals, such as Mn, was shown to modulate the function of these proteins (Hantke, 1987; Lee & Helmann, 2007). Furthermore, a range of physiological conditions such as oxidative (Nunoshiba et al., 1999) and acidic stress (Hall & Foster, 1996) was shown to affect Fur regulation.

The molecular mechanism by which iron mediates Fur interaction with DNA can be concluded from structural analysis of the large family of metal-dependant transcription regulators. It was concluded that a protein dimer is required to recognize a palindromic DNA sequence in an iron-dependant manner (Bes, Hernandez, Peleato, & Fillat, 2001). The structural support for this idea was provided first by crystallization of Fur from *P. aeruginosa* (Pohl et al., 2003). The surface of the protein is highly acidic, especially in the cavity formed at the interface of the two proteins (Sheikh & Taylor, 2009). These data, together with biochemical analysis, led to the proposal that this groove is the DNA-binding site (Ahmad, Brandsdal, Michaud-Soret, & Willassen, 2009). By structural modelling (Ahmad et al., 2009) and crystallographic analysis of the apo and holo form of the iron-dependant transcription factor PerR from *Bacillus subtilis* (Jacquamet et al., 2009), it was proposed that the regulatory mode for iron sensing is the reorganization of the dimeric structure (Fig. 3.8). In the absence of the metal, the structure becomes relaxed, which in turn reduces the binding activity of the transcriptional regulator. A recent computation study of Fur proteins from *Synechocystis* sp. PCC 6803 suggested that higher multimeric forms of these regulators should also be taken into account (Garcin et al., 2012).

Fe(II)-loaded Fur dimer recognizes a specific DNA sequence annotated as 'Fur box' (e.g. Hantke, 2001). The Fur box is a three forward–forward–reverse tandem hexamer with a recognition unit NAT(A/T)AT (Escolar, Perez-Martin, & de Lorenzo, 1998) and can be found in non-transcribed or (and) transcribed regions of genes. In the iron-loaded form, Fur adopts the V-shaped structure, binds to the Fur box leading to a block RNA polymerase binding (Faraldo-Gómez & Sansom, 2003). Thus, activation of iron starvation-dependant expression is induced by dissociation of Fur enforcing binding of the RNA polymerase to the according gene. In turn, the Fur-dependant activation of gene expression under normal iron levels is, in most cases, known so far indirectly manifested by repression of the sRNA RyhB, a Fur-repressed repressor (Massé, Salvail, Desnoyers, & Arguin, 2007; Mellin, Goswami, Grogan, Tjaden, & Genco, 2007). However, in some cases, only the iron-free Fur-apo form can bind to promoter regions, which represses gene expression under low iron concentrations as shown

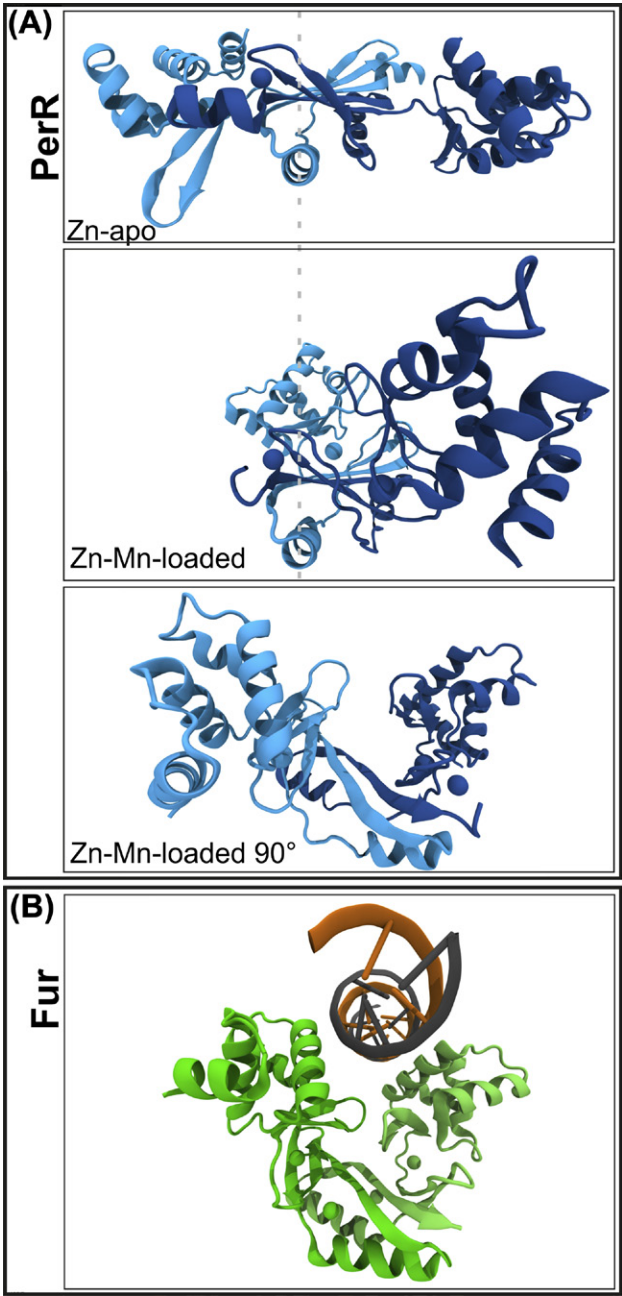


Figure 3.8 Fur family proteins

for the non-haeme-containing ferritin in *Helicobacter pylori* (Delany, Spohn, Rappuoli, & Scarlato, 2001). Recently, a fourth mode of Fur action was discovered by which the expression of the major iron storage protein of *E. coli*, FtnA, is regulated. In this case, repression of expression is manifested by the histone-like nucleoid-associated protein H-NS, which binds to the promoter region. H-NS is displaced by association of iron-loaded Fur, which enables binding of RNA polymerases (Nandal et al., 2010). Thus, multiple modes of Fur-dependant expression regulation exist, which all depend on the structural change of the protein upon iron interaction.

Fur proteins exist in cyanobacteria and have been described in, e.g. *Synechococcus* sp. PCC 7942 (Ghassemian & Straus, 1996), *Anabaena* sp. PCC 7119 (Bes et al., 2001), *Synechocystis* sp. PCC 6803 (Kunert, Vinnemeier, Erdmann, & Hagemann, 2003) and *Microcystis aeruginosa* (Martin-Luna, Hernandez, Bes, Fillat, & Peleato, 2006). A Fur box can be identified in targeted genes albeit with variable arrangement as compared to *E. coli* (Hernández, López-Gomollón et al., 2006). Similar to the autoregulatory system in *E. coli*, two putative Fur target sequences have been found upstream of the *fur* start codon, e.g. the *fur* gene of the filamentous cyanobacterium *Anabaena* sp. PCC 7119 (Bes et al., 2001). Thus, it can be concluded that the mode by which Fur regulates cyanobacterial gene expression is similar to the prokaryotic one.

Many cyanobacteria encode three Fur homologues. The interplay between these factors was analysed in *Anabaena* sp. PCC 7120, where they are termed FurA (*all1691*; *sll0567* in *Synechocystis* sp. PCC 6803), FurB (*all2473*; *sll1937* in *Synechocystis* sp. PCC 6803) and FurC (*alr0957*; not yet identified in *Synechocystis* sp. PCC 6803; Hernández, López-Gomollón, Bes, Fillat, & Peleato, 2004; Kaneko et al., 1996). FurA is essential (Hernández, López-Gomollón et al., 2004; Hernández, Muro-Pastor et al., 2006; Kunert et al., 2003), shows the highest similarity to Fur from heterotrophic bacteria and contains the characteristic HHXHXXCXXC motif. FurB contains a cluster of three histidine residues, instead of the HHXHXXCXXC motif (Kunert et al., 2003). In contrast to FurA, the function of FurB is not essential (Napolitano et al., 2012). An additional Fur family regulator is PerR (*slr1738*), usually thought of as a regulator of oxidative stress. However, in *Synechocystis* sp. PCC 6803, this protein was found to play a role in iron homeostasis (Li et al., 2004; Shcolnick, Summerfield, Reytman, Sherman, & Keren, 2009) and in the response to heavy metals (Houot et al., 2007) as well.



#### 4.1.1. *FurA – a regulator of global transcription?*

At the transcriptional level, FurA of *Anabaena* sp. PCC 7120 is autoregulated, as established for *Anabaena* sp. PCC 7119 (Hernández, López-Gomollón *et al.*, 2006), and likely modulated by FurB and FurC. The latter was concluded from the observed strong binding of FurB and the weak binding of FurC to the promoter region of FurA (Hernández, López-Gomollón *et al.*, 2004). The activity is influenced by manganese and the redox state of the environment (Hernández, López-Gomollón *et al.*, 2006), the latter influencing the oligomerization state of the protein (Lostao, Peleato, Gómez-Moreno, & Fillat, 2010). FurA regulated genes were analysed in strains with enhanced FurA content either by disrupting the sRNA (Hernández, Muro-Pastor *et al.*, 2006) or by placing *furA* behind a copper-inducible promoter (Gonzales *et al.*, 2010). The latter mutant shows a fragmentation phenotype as previously identified for strains with defects in the outer membrane integrity or septosome formation (Bauer, Buikema, Black, & Haselkorn, 1995; Flores *et al.*, 2007; Nicolaisen, Mariscal *et al.*, 2009; Wilk *et al.*, 2011). This is consistent with the observed FurA-dependant regulation of the outer membrane TBDT SchT (González *et al.*, 2010).

Enhanced levels of FurA are found to reduce the expression of genes coding for the DNA-binding protein A from stationary phase DpsA (alr3808; Hernández *et al.*, 2007), the 11-kDa protein of PSII PsbZ (all1258), the iron-containing superoxide dismutase SodB (alr2938), the thiol peroxidases GCT1 (alr3183) and GCT3 (all2375; Gonzales *et al.*, 2010), peroxiredoxins (alr4641; all1541), the thioredoxin reductase TrxB (all0737), the modulators of DNA gyrase TldD (all5219) and PmbA (all5218), the protease Abp1 (all1940), the succinate-semialdehyde dehydrogenase (all3556) and the two-component regulator OrrA (alr3768; Gonzales *et al.*, 2011). Confirming a FurA regulation by repression, FurA was found to physically interact with almost all of the promoter regions of the according genes (González *et al.*, 2010, 2011). Remarkably, several genes upregulated in FurA expression strains have been identified as well. Among those are genes coding for the PSI components such as PsaA (alr5154) and PsaB (alr5155), for the PSII component PsbB (all0138), for the manganese-containing superoxide dismutase SodA (alr2938), for the bacterial actins MreB and MreC (all0087; all0086), for the IsiA protein (all4001), for the outer membrane TBDT SchT (alr0397; Gonzales *et al.*, 2010), for the thiamine biosynthesis protein ThiC (all0982) and the CO<sub>2</sub> concentrating mechanism protein CcmM (all0865; Gonzales *et al.*, 2011). Thus, one could speculate that FurA not only is involved in expression repression under normal conditions but



also appears to be a fairly global transcriptional regulator important for cellular function. However, the interpretation whether these are genes targeted by the apo-FurA, whether they are targets of FurB, which is downregulated in the according strain, or whether the observations correspond to pleiotropic effects caused by a possible iron limitation as a consequence of the overexpression of an iron-binding protein remains largely unknown. For the promoter regions of *isiA*, *schT*, *mreBCD*, *thiC* and *cmM*, a direct interaction of FurA with the promoter region was documented, while for *sodA*, such interaction could not be documented. Thus, it will be of importance to explore the transcription level, in general, under different conditions to fully uncover the functionality of FurA.

#### 4.1.2. *FurB and FurC*

Initially, it was speculated that FurB might act as iron-response regulator (Irr)-like protein as FurB is more active in the absence of iron and shows a putative haeme regulatory motif near its C-terminal sequence (CPVHNN; Nicolaisen & Schleiff, 2010). However, recent work proposed two at least additional functions. It was demonstrated that *furB* and *furC*, in contrast to *furA*, are induced by oxidative stress enforced by H<sub>2</sub>O<sub>2</sub> treatment (López-Gomollón, Sevilla, Bes, Peleato, & Fillat, 2009). In addition, FurB expression in *E. coli* results in a reduced sensitivity to oxidative stress and *in vitro* DNA-protection studies revealed a protective function of FurB (López-Gomollón et al., 2009). On the basis of this observation, it was proposed that FurB acts as a DPS protein (López-Gomollón et al., 2009). In parallel, FurB, but not FurA or FurC, was found to specifically bind to promoter regions of genes in a cluster encoding several metalloproteins, and this interaction was found to be zinc dependant (Napolitano et al., 2012). By *in vitro* binding studies, a DNA element defined by TGATAATXATTATCA was discovered (Napolitano et al., 2012). At least 33 putative FurB-binding sites were identified in the genome of *Anabaena* sp. PCC 7120. The subsequent analysis revealed a strong regulation of at least seven different operons, namely *alr1197–alr1199*, *all1751–all1750* and *all4729–all4721* coding for metalloproteins, as well as *alr3242–alr3243*, *alr4028–alr4031*, *all0833–all8032* and *all0830*, which code for an ABC transporter, a periplasmic zinc-binding protein (*all0833*; ZnuA) and a TonB-dependent outer membrane transporter (*alr3242*, HutA; *alr4028–alr4029*; BtuB; Mirus et al., 2009). Thus, FurB might have overlapping functions in sensing oxidation, iron and zinc. Alternatively, it might be that FurB is indeed a Zur as recently suggested (Napolitano et al., 2012). The interaction of FurB with the promoter region of *furB* was inhibited

in the presence of zinc (Hernández, López-Gomollón *et al.*, 2004) and it remains to be analysed whether this supports the multiple modes of FurB operation or reflects an apo-FurB function of the zinc-specific transcription factor on its own promoter.

The least is known about the function of FurC. FurC only seems to be involved in the regulation of the binding activity of FurA and FurB to corresponding promoter regions, rather than to bind itself to any fur-type promoter (Hernández, López-Gomollón *et al.*, 2004). Its expression is induced by the addition of H<sub>2</sub>O<sub>2</sub> indicative of an importance in response to oxidative stress. However, expression in *E. coli* did not lead to an alteration of the response to oxidative stress (López-Gomollón *et al.*, 2009). Thus, its molecular action is still unknown and remains to be established.

Recent analysis of the expression of furA, furB and furC in *M. aeruginosa* uncovered a differential expression of these genes during exponential and stationary phase (Alexova *et al.*, 2011). Lowering the iron concentration to 100 nM enforced a downregulation of furA and furB only during the stationary phase and only in the toxic *M. aeruginosa* sp. PCC 7806. In contrast, when the iron concentration was further reduced to 10 nM, a downregulation of all three genes was observed in the stationary phase in toxic form. The differential regulation of Fur proteins in the two strains might be explained by the importance of Fur for the expression of the microcystin biosynthesis gene cluster (Martin-Luna, Sevilla *et al.*, 2006). In addition, an upregulation of furB and furC was observed under exponential growth in both the toxic strain *M. aeruginosa* sp. PCC 7806 and in the nontoxic strain *M. aeruginosa* sp. PCC 7005, while expression of furA is not affected under these conditions (Alexova *et al.*, 2011). This result is in line with the suggested regulation of the expression of furB and furC by the redox state rather than by the iron content and thus, FurA appears to be the main regulator for iron starvation response.

#### **4.1.3. Communication between the Cyanobacterial Fur and NtcA Systems**

Certain cyanobacterial species, both single cell and filamentous heterocyst-forming, fix atmospheric nitrogen using nitrogenase – an Fe<sub>7</sub>Mo-containing enzyme. NtcA is the central transcription factor in the response to nitrogen limitation in all cyanobacteria. In *Anabaena* sp. PCC 7120, NtcA plays an important role in the regulation of heterocyst development and nitrogen fixation (Nicolaisen, Hahn, & Schleiff, 2009; Ohashi *et al.*, 2011). A coordination in iron acquisition and nitrogen metabolism might be manifested

by the NtcA-dependant activation of *pkn41* and *pkn42* expression under low iron conditions (Cheng, Shi, Latifi, & Zhang, 2006). These two proteins, with Ser/Thr kinase and His kinase domains, are essential under low iron or nitrogen conditions. However, the downstream signal transduction cascade has not yet been described. The *pkn22* operon, including the gene of the protein kinase Pkn22, is regulated by oxidative stress and iron starvation (Latifi, Ruiz, Jeanjean, & Zhang, 2007; Xu, Jeanjean, Liu, & Zhang, 2003) and might be under the control of NtcA as well (Latifi, Ruiz, & Zhang, 2009), a statement, which, however, is not yet further explored.

Another mode of genetic interaction is the strong activation of P<sub>fur</sub>A in proheterocysts and heterocysts as a result of NtcA binding to operators present in the upstream region of *furA* and *alr1690- $\alpha$ -furA* (López-Gomollon, Hernández, Wolk, Peleato, & Fillat, 2007). Consistently, the promoter of *furA* shows several putative NtcA-binding sites. Thus, *furA* expression, and thereby its subsequent regulatory function, is dependant on the nitrogen status as well (Hernández, Peleato, Fillat, & Bes, 2004; López-Gomollon, Hernández, Pellicer et al., 2007; López-Gomollon, Hernández, Wolk et al., 2007).

Thus, two modes for the integration of iron and nitrogen starvation exist. On the one hand, signal transduction cascade components involved in the response to oxidative stress or iron starvation are linked to nitrogen fixation as well. On the other hand, the expression of 'photosynthetic genes' is regulated by FurA and NtcA. This link is a logical consequence considering the iron demand of the nitrogenase and the sensitivity of this enzyme to oxidation leading to a regulatory network able to respond to a range of environmental conditions. However, the network is not yet fully understood and described. For instance, one *exbB* gene (see above section 2.4) of *Anabaena* sp. PCC 7120 is strongly induced during nitrogen starvation (Stevanovic et al., 2012), which suggests a coupling between nitrogen starvation and enhanced iron uptake even at the transport level.

#### 4.2. Iron-Dependant Regulation of Transcription by Antisense RNA

A major regulatory pathway includes the post-transcriptional control of *furA*. The *alr1690- $\alpha$ -furA* antisense RNA interferes with the *furA* transcript as first documented for *Anabaena* sp. PCC 7120 (Hernández, Muro-Pastor et al., 2006). The *cis- $\alpha$ -fur* RNA also exists in *M. aeruginosa* PCC 7806 and *Synechocystis* sp. PCC 6803 although the genomic context differs (Sevilla et al., 2011). In *M. aeruginosa*, *fur* appears to be part of a dicistronic

operon with a *cis*- $\alpha$ -sufE RNA regulating SufE abundance. SufE is a part of the SufBCDE system involved in Fe-S cluster assembly in cyanobacteria (Balasubramanian *et al.*, 2006; Seki *et al.*, 2006). Thus, this antisense  $\alpha$ -sufE RNA encoded by the same genomic region as FurA would form a link between Fur- and SufE-mediated regulation in *M. aeruginosa*. The  $\alpha$ -fur RNA in *M. aeruginosa* is tightly regulated by oxidative stress as its transcript was highly induced upon addition of hydrogen peroxide (Martin-Luna *et al.*, 2011). The latter might reflect the regulatory mechanism observed for the Fur-dependant expression of the microcystin biosynthesis gene cluster (Martin-Luna, Sevilla *et al.*, 2006). However, the change of expression of the microcystin biosynthesis gene cluster is rather dependant on a functional electron transfer chain than reactive to oxidative stress (Sevilla, Martin-Luna, Bes, Fillat, & Peleato, 2012).

However, the *cis*- $\alpha$ -fur RNA is not the only antisense RNA involved in the regulation of iron level responses. *IsiR* is an internal antisense RNA (Düh-ring, Axmann, Hess, & Wilde, 2006), which regulates the expression of *isiA* (see below section 4.3.1). The expression of *isiR* creates a temporal delay that results in substantial *isiA* transcript accumulation only after sustained iron limitation (Legewie, Dienst, Wilde, Herzel, & Axmann, 2008).

Currently, three antisense RNA involved in the regulation of iron starvation are discovered. However, as only the *cis*- $\alpha$ -fur RNA was generally identified while other antisense RNAs appeared to be species specific, it is likely that further antisense RNAs involved in the regulation of iron starvation might exist. Furthermore, recent transcriptome profiling uncovered a wealth of expressed antisense and other noncoding RNAs in *Synechocystis* sp. PCC 6803 (Mitschke *et al.*, 2011). Thus, transcriptomic studies and high-throughput profiling under different conditions, including iron starvation, will undoubtedly uncover additional global and species specific regulatory noncoding RNAs.

### 4.3. Physiological Responses

Iron starvation is linked to a number of physiological responses. It is well documented that photosynthetic apparatus activity is reduced on a chlorophyll basis (Ivanov *et al.*, 2000). The organization of the photosynthetic apparatus changes as well. In the early stages of Fe limitation, Photosystem I trimers are monomerized and the capacity for state transition is reduced (Ivanov *et al.*, 2006). In parallel, IsiB (flavodoxin) replaces ferredoxin and *isiA* is expressed to high levels (discussed below in Section 4.3.2). In addition, transitions in or out of iron limitation result in enhanced oxidative stress. The relationship

between oxidative stress and iron starvation was described in a number of studies (Houot et al., 2007; Li et al., 2004; Shcolnick et al., 2009). The data presented in these studies suggest that intracellular Fe status is a major determinant of oxidative stress, possibly through Fenton-type reactions with free iron. The response seems to be co-ordinated by the function of PerR-type regulators and involves the activity of DPS-type proteins, probably as iron scavengers reducing the risks of intracellular Fenton reactions.

Several transcriptomic studies were performed on the transition from replete to limiting iron conditions (Shcolnick et al., 2009; Singh, McIntyre, & Sherman, 2003 in *Synechocystis* sp. PCC 6803; Nodop et al., 2008 in *Synechococcus elongatus* PCC 7942). Common to all these studies is a concerted decrease in photosynthesis and respiration genes. The *isiAB* operon is the major upregulated operon. Both effects fit well with the physiological data. Additional upregulated genes include components of the iron transport system (FuhA and FutA2, for example, Shcolnick et al., 2009) and of the iron sulfur cluster assembly system (*sufC*, *sufD*, *sufS*; Nodop et al., 2008). In *S. elongatus* PCC 7942, specific transcriptional responses in *idi* genes were observed. The *idiA* gene, the transcription factor coding *idiB*, and the iron-deficiency-induced gene C, *idiC*, were drastically induced (Nodop et al., 2008). The function of some of the induced genes in iron-deficiency response has been studied and will be discussed below. However, it is important to note that many unknown and hypothetical proteins were affected by this treatment, suggesting a more extensive and complicated response than we can currently decipher.

#### 4.3.1. The *IsiA* Protein

IsiA is present in the genome of many cyanobacterial species of all five cyanobacterial branches (Geiss et al., 2001; Nicolaisen & Schleiff, 2010). The expression of *isiA* is induced by iron starvation (Geiss et al., 2001; Kouril et al., 2005; Latifi et al., 2005). Initially, it was discussed that its expression is the result of Fur-mediated derepression of transcription (Ghassemian & Straus, 1996). Interestingly, *isiA* expression is significantly upregulated in the *furA* overexpression strain (González et al., 2010). This might be related to the observed enhanced expression of *isiA* in response to oxidative stress (Michel & Pistorius, 2004) and thus, the induction of *isiA* expression by other stresses might reflect a function in photosystem protection in general.

*isiA* codes for the CP43' protein, which forms a chlorophyll-protein complex. Originally, it was identified as the protein responsible for the blue shift in the chlorophyll peak and increased 682-nm fluorescence in

iron-limited cyanobacteria (Burnap *et al.*, 1993). Based on that analysis, it was suggested that CP43' can be either an additional antenna complex or a scavenging protein involved in the relocation of chlorophyll. Structural analysis demonstrated that it forms a ring around trimeric and monomeric PSI (Bibby, Nield, & Barber, 2001; Boekema *et al.*, 2001) and thus, it is assumed that CP43' forms an alternative antenna complex for PSI, increasing the functional absorption cross-section for PSI. On the other hand, it was suggested that CP43' acts as a quencher protecting the photosynthetic apparatus from photo-inhibitory damages. This suggestion is strengthened by spectroscopic studies demonstrating fluorescence quenching in CP43' complexes (van der Weij-de Wit, Ihalainen, van Grondelle, & Dekker, 2007). The presence of *isiA* genes is currently being explored as a biomarker for iron limitation in oceans (Richier *et al.*, 2012).

#### 4.3.2. The Flavodoxin *IsiB*

The soluble electron transfer protein ferredoxin is required for the transfer of electrons from PSI to ferredoxin-NADP<sup>+</sup> reductase. The protein contains a [2Fe-2S] cluster as a cofactor and is one of the major iron-containing proteins in cyanobacterial cells. Consequently, under iron-limiting conditions, the function of this protein is compromised. To avoid malfunction, these proteins are replaced by flavodoxin under such conditions (Kouril *et al.*, 2005; Latifi *et al.*, 2005; Laudenbach *et al.*, 1988). The replacement of ferredoxin by flavodoxin defines the flavodoxin/ferredoxin ratio as a marker for the iron status of a cyanobacterial cell (Doucette, Erdner, Peleato, Hartmann, & Anderson, 1996). However, flavodoxins have not been identified in all cyanobacteria. For example, *Anabaena* ATCC 29211 lacks the potential to synthesize flavodoxin (Sandmann, Peleato, Fillat, Lazaro, & Gomez-Moreno, 1990). In this cyanobacterium, the ferredoxin content is decreased at very low iron concentrations (below 1  $\mu$ M) without replacement.

Flavodoxin is encoded by *isiB*, downstream of *isiA* and on the same operon. It contains a flavin cofactor and, thus, does not require iron. The functionality and efficiency of flavodoxin is comparable to that of ferredoxin. This can be explained by the ferredoxin-like oxidation/reduction potential of the semiquinone/hydroquinone pair (Fillat, Edmondson, & Gomez-Moreno, 1990; Fillat, Sandmann, & Gomez-Moreno, 1988). However, flavodoxin is not only important under iron-limiting conditions. For example, *isiB* is constitutively expressed in heterocysts where the flavodoxin is thought to function in the transport of electrons to the nitrogenase (Sandmann *et al.*, 1990). Furthermore, it has been shown in *Synechocystis* sp.

PCC 6803 that flavodoxin expression is not able to fully compensate for a deletion of the ferredoxin-encoding gene (Poncelet, Cassier-Chauvat, Leschelle, Bottin, & Chauvat, 1998).

#### 4.3.3. *IdiABC*

Several additional iron-deficiency proteins were identified. Iron-deficiency-induced protein A was identified in *S. elongatus* PCC 7942 (*idiA*; Michel et al., 1998). This gene is specific for cyanobacteria and cannot be found in green algae or higher plants (Michel & Pistorius, 2004). The expression of *idiA* is under the control of the transcriptional activator IdiB (Michel, Pistorius, & Golden, 2001), which is induced upon iron starvation but disappears shortly after iron depletion (Yousef, Pistorius, & Michel, 2003). The transcript of *idiB* is more stable than the transcript of *isiAB* and remains detectable for more than 10 h after iron depletion (Yousef et al., 2003). Electron microscopic analysis of single PSII particles indicated that IdiA directly interacts with CP43 and D1 (Lax et al., 2007). Thus, a model was suggested, by which IdiA protects the cytosolically exposed acceptor side of PSII exposed after phycobilisome degradation (Nodop et al., 2008).

However, in the literature, 'IdiA' was also used for the gene product of *slr1295* in *Synechocystis* sp. strain PCC 6803 (Tölle et al., 2002). Indeed, the latter is the closest homologue when the IdiA sequence from *S. elongatus* PCC 7942 (*syc1920\_d*) is used for a search. However, reblast analysis using the sequence from *Synechocystis* sp. strain PCC 6803 reveals a closer relation to *syc0146\_c*, which encodes an iron transport system substrate-binding protein. This is confirmed by the relation to *alr1382* from *Anabaena* sp. PCC 7120, which has been annotated as periplasmic iron transport protein FutA (Stevanovic et al., 2012). Considering these results, the *Synechocystis* IdiA should be considered as FutA1, the second annotated name of the protein (Katoh, Hagino, & Ogawa, 2001).

IdiB is a helix–turn–helix transcriptional regulator of Crp/Fnr type (Michel & Pistorius, 2004). It regulates not only *idiA* but also other genes involved in protection against damage caused by iron limitation. In a global analysis of iron starvation-dependant expression in wild-type *S. elongatus* PCC 7942, an *idiB*-free mutant and a merodiploid mutant with significantly reduced expression *idiB* and *idiC* revealed a global function for IdiB. For example, expression of *irpA*, *irpB*, *somA*(2), *somB*(1), *idiA*, as well as genes coding for an acetate kinase (*ackA*) and for phosphoglycerate mutase (*pgmA*). The latter two are both proposed to function in acetate–phosphate metabolism (Nodop et al., 2008) and thus, the IdiB-dependant regulation of



ackA/pgam was suggested as evidence for a substrate-level phosphorylation under iron-limiting conditions via acetyl phosphate to acetate conversion, but the experimental proof for such pathway is still missing.

A third iron-regulated gene, *idiC*, is positioned upstream of *idiA* and *idiB* in *S. elongatus* PCC 7942 (Pietsch, Staiger, Pistorius, & Michel, 2007). *IdiC* is a member of the thioredoxin-like ferredoxin family and was suggested to function in protecting the photosynthetic activity in a manner similar to NuoE, a peripheral subunit of the *E. coli* NDH-1 complex (Pietsch *et al.*, 2007). However, as seen for *IsiB*, the function of *IdiC* is not restricted to iron starvation response as the gene is essential. The latter is in agreement with the recent observation that *idiC* expression is enhanced during stationary phase (Pietsch *et al.*, 2011). Based on these results, it was suggested that *IdiC* might function in the electron transport process from NAD(P)H to plastoquinone (Pietsch *et al.*, 2011). Thus, *IdiC* is most likely a factor involved in the global regulation of photosynthesis.

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# Functional Genomics of Metalloregulators in Cyanobacteria

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## Abstract

Cyanobacterial metabolism relies on the activity of many enzymes and other proteins containing metal-rich cofactors that are absent in nonphotosynthetic organisms. Most of those micronutrients play key roles in or are associated to photosystems, respiratory electron transport chains and many enzymes involved in nitrogen metabolism. Since

metal homeostasis is crucial for the ecological success of cyanobacteria, trace metal bio-uptake is strictly regulated by a number of metal-sensor proteins and regulatory proteins that often also contain metals. This chapter discusses functional studies undertaken to-date from a genomic point of view, as well as the main structural and mechanistic insights into the major families of metalloregulators in cyanobacteria. Reverse genetics, transcriptomics and other assays used for the identification of metal-regulated genes reveal interesting connections between metabolic networks and interactivity between major regulons. These data provide a better understanding of cyanobacterial physiology including maintenance of metal homeostasis, strategies to deal with different stresses and the basis of cyanotoxicity.



## 1. INTRODUCTION

Transition metals are essential components of all living cells. They act as main cofactors for oxidation–reduction reactions in electron transfer chains, in hydrolytic and acid–base chemistry and are key structural elements that stabilize protein fold. Cyanobacterial metabolism relies on the activity of many enzymes and other proteins that contain metal-rich cofactors that are absent in nonphotosynthetic organisms. Most of these micro-nutrients play key roles in or are associated to photosystems, for example, manganese in the water-splitting oxygen-evolving complex, magnesium in chlorophyll and copper in plastocyanin. Besides, biological nitrogen fixation needs a large amount of metals, such as the nitrogenase complex with molybdenum (or vanadium) and iron. For these reasons, cyanobacterial metal requirements are greater than in other prokaryotes. In particular, the high content of iron present in the machineries involved in photosynthesis and nitrogen assimilation makes cyanobacteria highly dependent on iron, whose needs are one order of magnitude larger than those in heterotrophic bacteria (Shcolnick & Keren, 2006).

During their evolution, cells develop a broad network of metalloregulatory proteins involved in transport, metal trafficking and metal homeostasis and resistance to deficiency, as well as an efficient cross-talk with other regulatory networks. In fact, the effectiveness of these systems confers on organisms an important adaptive advantage, which is very clear in the case of iron metabolism (Straus, 1994). In many cases, the ability of a micro-organism to capture and incorporate metals determines its ecological success. In fact, cyanobacteria developed, during evolution, very efficient mechanisms for maintaining metal homeostasis.

Metalloregulation in cyanobacteria is mainly carried out by metal-sensor proteins and regulatory proteins containing metals. In recent years,

their characterization has contributed significantly to providing important insights into how bacteria handle metals and harmonize many aspects of their metabolism. The high sensitivity and selectivity of metalloregulatory proteins has been explained on a molecular basis by solving the crystal structures of representatives from the main families involved in this important function (Cook, Kar, Taylor, & Hall, 1998; Pohl et al., 2003).



## 2. GENERAL FEATURES OF METALLOREGULATORS

Metal-sensor proteins are usually allosteric proteins whose reversible interaction with the regulatory metal drives conformational changes that affect DNA binding. They exhibit metal-binding and DNA-binding domains and, in some cases, possess an additional structural metal (Dian et al., 2011; Lucarelli et al., 2007). Usually, DNA-binding proteins act as a dimer or a dimer of dimers. In some cases, metal sensors with different metal specificities may interact (Fleischhacker & Kiley, 2011; Reyes-Caballero, Campanello, & Giedroc, 2011).

Residues involved in metal binding are highly conserved. Some metal-sensor proteins can also be involved in other functions apart from transcriptional regulation. This potential multifunctionality of metalloregulators is a challenging issue for future investigation aimed at better understanding of cell physiology.



## 3. MAIN FAMILIES OF METALLOREGULATORS IN CYANOBACTERIA

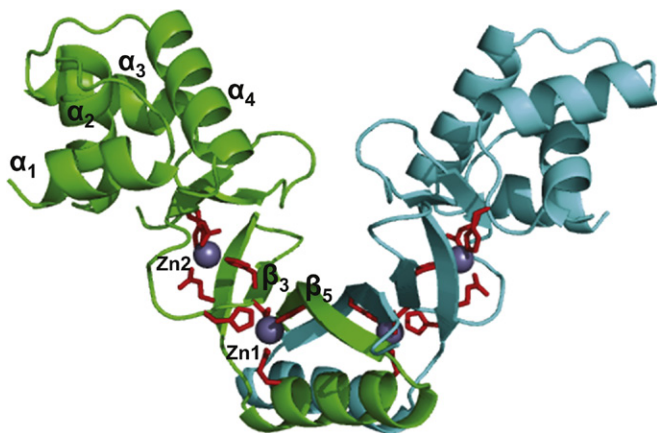
### 3.1. The Fur Superfamily

Ferric Uptake Regulator (Fur) proteins are among the most ubiquitous regulators in prokaryotic organisms. Initially identified as iron-sensing repressors in heterotrophic bacteria, Fur was the first member characterized and the protein that gives its name to the family. It is assumed that Fur works as a classical repressor using  $\text{Fe}^{2+}$  as a cofactor to negatively regulate expression of their target genes. However, it is now known that Fur proteins display an ample diversity in metal selectivity and biological functions (Lee & Helmann, 2007), including sensors of iron (Fur), zinc (Zur), manganese (Mur) and nickel (Nur). Other family members use metal-catalysed oxidation reactions to sense peroxide stress (PerR) or the availability of haeme (Irr). Thus, Fur proteins are involved in the control of a large number of

genes involved in general metabolism, electron transport, virulence and defence against different stresses.

### 3.1.1. Structural features of Fur proteins

Although Fur-like proteins have a wide diversity of metal selectivity and biological functions, they share a similar architecture that serves to better understand the activation and metal discrimination mechanism of this group of proteins. The first crystal structure for a Fur homologue showed a basic folding with two well-defined domains: an N-terminal DNA-binding domain and a C-terminal dimerization domain (Pohl *et al.*, 2003). The DNA-binding domain of Fur from *Pseudomonas aeruginosa* was composed of four helices followed by a two-stranded antiparallel  $\beta$ -sheet displaying a winged helix–turn–helix (HTH) motif (Fig. 4.1). The dimerization domain of each Fur monomer consisted of two structural elements that were involved in forming the functional protein dimer, an  $\alpha/\beta$ -domain and a long  $\alpha$ -helix covered by three antiparallel  $\beta$ -strands from the first element. Although this main folding has been confirmed by X-ray crystallography of other members of the Fur family of metalloregulators, such as *Helicobacter pylori* Fur (Dian *et al.*, 2011), *Streptomyces coelicolor* Nur (An *et al.*, 2009), *Mycobacterium tuberculosis* FurB/Zur (Lucarelli *et al.*, 2007) or *Bacillus subtilis* PerR (Jacquamet *et al.*, 2009), there are structural differences that may be responsible for some exceptions in the regulatory capacity observed in this protein family (Dian *et al.*, 2011; Hernández, Bes, Fillat, Neira, & Peleato, 2002).



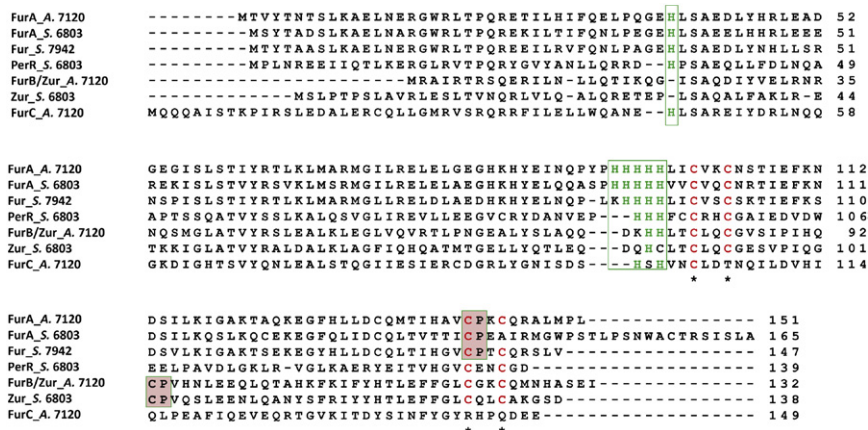
**Figure 4.1** Three-dimensional model of *Pseudomonas aeruginosa* Fur generated by PISA Server (Krissinel & Henrick, 2007) and drawn using PyMOL (Delano, 2006). Several chains of amino acids discussed in the text are shown in darker grey. For colour version of this figure, the reader is referred to the online version of this book.



No crystal structure is available for cyanobacterial Fur homologues, though their main folding is probably shared with other Fur proteins. In fact, FurA from *Anabaena* PCC 7120 shows around 40% sequence similarity with *Pseudomonas* Fur and has similar helical content, as demonstrated by FTIR and far-UV CD (Hernández et al., 2005). A three-dimensional model for the FurA monomer from *Anabaena* PCC 7120 has been obtained by homology modelling based on its similarity with *P. aeruginosa* Fur, although the lack of strong sequence identity at the dimerization interface between *P. aeruginosa* Fur and FurA precluded dimer modelling (Hernández et al., 2005). Homology modelling has also been used to build the 3D structure of the PerR-like regulator Slr1738 from *Synechocystis* PCC 6803 (37% homology with *P. aeruginosa* Fur protein) and its target DNA (Garcin et al., 2012).

### 3.1.1.1. Metal-binding site

Fur proteins show two potential metal-binding motifs rich in histidines and cysteines; a conserved HHXHX<sub>2</sub>CX<sub>2</sub>C and another, less conserved, carboxyl-terminal motif CX<sub>2</sub>C (Fig. 4.2). The structure of Fur from *P. aeruginosa* exhibits two metal-binding sites (Pohl et al., 2003). Site 1, placed in the dimerization domain, comprises the side chain of residues His<sup>86</sup>, Asp<sup>88</sup>, Glu<sup>107</sup> and His<sup>124</sup> and a water molecule resulting in a distorted



**Figure 4.2** Alignment of a representative subset of different members of the Fur family from cyanobacteria. A. 7120, *Anabaena* PCC 7120; S. 6803, *Synechocystis* PCC 6803; S. 7942, *Synechococcus* PCC 7942. The conserved histidine in the N-terminal domain potentially involved in DNA-binding and the His-rich motif are boxed. Cysteine residues in the CXXC redox motifs are indicated with asterisks. Haeme-regulatory CP motifs are indicated in grey boxes. For colour version of this figure, the reader is referred to the online version of this book.



octahedral geometry. Site 2 connects the DNA-binding domain with the dimerization domain including the side chain of residues His<sup>32</sup>, Glu<sup>80</sup>, His<sup>89</sup> and Glu<sup>100</sup> with a tetrahedral geometry (Fig. 4.1). The role of sites 1 (regulatory) and 2 (structural) for *Pseudomonas* Fur is subject to controversy. Some authors propose that Fur from *P. aeruginosa* detects Fe<sup>2+</sup> through the site initially described to accommodate the structural Zn<sup>2+</sup> (site 2) but according to biochemical analyses, it lacks structural Zn<sup>2+</sup>. Site 1 could be a metal-binding site of low affinity without biological significance (Lee & Helmann, 2007).

The presence of a structural Zn<sup>2+</sup>-binding site has been found in most Fur proteins crystallized to date (Dian *et al.*, 2011; Jacquamet *et al.*, 2009; Lucarelli *et al.*, 2007; Sheikh & Taylor, 2009). This structural site usually represents a regular tetrahedral co-ordination of four cysteine residues belonging to two CX<sub>2</sub>C motifs. However, the presence of such motifs does not seem to ensure the binding of structural zinc. In fact, Cys4–Zn appears not to be essential for maintaining the DNA competent conformation and, hence, for the DNA-binding activity of Nur (An *et al.*, 2009). This also seems to be the case of recombinant cyanobacterial FurA. Despite having two pairs of CX<sub>2</sub>C (Cys<sup>101</sup>, Cys<sup>104</sup>, Cys<sup>141</sup> and Cys<sup>144</sup>), metal analysis and electrospray ionization MS evidenced that neither zinc nor other metals are present in this Fur homologue (Hernández *et al.*, 2002). However, these motifs are probably important in the cyanobacterial FurA regulatory mechanism since Cys<sup>101</sup> and Cys<sup>141</sup> correspond to residues Cys<sup>93</sup> and Cys<sup>133</sup> present in the dimerization domain in *Vibrio cholerae* Fur. They are connected by a disulphide bond, which plays an analogous role to that of the salt bridge between Asp<sup>94</sup> and Arg<sup>131</sup> that stabilizes the β3–β5 antiparallel β-sheet in each subunit of the *Pseudomonas* Fur dimer (Sheikh & Taylor, 2009), and Cys<sup>141</sup> has been found to be involved in haeme co-ordination by cyanobacterial FurA (Pellicer *et al.*, 2012).

The metal-binding site that binds the regulatory metal seems to be conserved in all Fur and Fur-like proteins although it shows some variability in its co-ordination depending on the Fur homologue and the metal. However, it always involves a histidine residue from the loop between α2 and α3 in the DNA-binding domain (Fig. 4.1). This histidine is also conserved in most cyanobacterial FurA, FurC (Fig. 4.2) and Slr1738 (PerR) (Garcin *et al.*, 2012). In FurA from *Anabaena* PCC 7120, it corresponds to His<sup>39</sup> that according to a monomer, three-dimensional model belongs to a buried core formed by polar residues His<sup>39</sup>, His<sup>85</sup>, His<sup>96</sup>, His<sup>98</sup>, Glu<sup>87</sup> and Glu<sup>109</sup> (Hernández *et al.*, 2005). This histidine is noticeably absent

in cyanobacterial FurB/Zur orthologues (López-Gomollón, Sevilla, Bes, Peleato, & Fillat, 2009 and Fig. 4.2).

#### 3.1.1.2. DNA-binding sites

Under conditions of iron abundance, iron-bound Fur dimers bind to target promoters in their Fur boxes, also called iron boxes. Fur-binding sequences were originally regarded as 19-bp inverted repeats with the consensus (GATAATGATAATCATTATC), and reinterpreted as arrays of NATA/TAT heptamers (Escolar, Pérez-Martín, & de Lorenzo, 1999). Binding of FurA from *Anabaena* to their DNA targets is enhanced in the presence of divalent metals and reducing conditions (Hernández, López-Gomollón et al., 2006). Moreover, cyanobacterial FurA binds haeme and this interaction negatively affects its in vitro DNA-binding ability (Hernández, Peleato, Fillat, & Bes, 2004). Footprinting assays using *furA* and flavodoxin promoters led to the identification of the first experimentally defined binding sites for a cyanobacterial FurA protein. These Fur boxes consist of arrays of A/T-rich sequences that show a faint homology with the *Escherichia coli* consensus sequence. Furthermore, experimental work suggests that, in addition to proper A/T arrays, DNA conformation might be an important factor in FurA target recognition and binding (González, Bes, Peleato, & Fillat, 2011). These features are somewhat different from the DNA-binding properties found for the other cyanobacterial Fur paralogues (Hernández, López-Gomollón, Bes, Fillat, & Peleato, 2004; López-Gomollón et al., 2009; Napolitano et al., 2012).

#### 3.1.2. Occurrence and functions of Fur paralogues in cyanobacteria

The isolation of a *fur* gene in *Synechococcus* PCC 7942 through an *E. coli*-based in vivo repression assay was the first evidence of the existence of a Fur protein in cyanobacteria (Ghassemian & Straus, 1996). In this cyanobacterium, deletion of *fur* resulted in meridioids that showed iron-deficiency symptoms in iron-replete medium, as well as partial derepression of flavodoxin and of hydroxamate siderophores. Advances in whole genome sequencing of diverse cyanobacterial strains allow identification of this essential *fur* gene as Synpcc7942\_0987. Moreover, searching for ORFs exhibiting the His-rich motif characteristic of Fur proteins across the 39 cyanobacterial genomes available in the cyanobase (<http://genome.kazusa.or.jp/cyanobase>) (Nakamura, Kaneko, Hirose, Miyajima, & Tabata, 1998) reveals the presence of several Fur paralogues in all the strains sequenced to date. Although three Fur-like proteins are commonly found in most cyanobacteria, some

*Prochlorococcus* strains, such as SS120 or MED4, harbouring the smallest cyanobacterial genomes contain only two *fur* genes, in contrast with *Acaryochloris marina* MBIC11017 that exhibits the highest number of Fur and Fur-like paralogues, namely five Fur-like, four PerR-like and four Zur-like members (Table 4.1). This scenario suggests that this family of transcriptional regulators presents a functional specialization in cyanobacteria though microarray-based analysis has also evidenced redundant functions and cross-talking between Fur proteins.

### 3.1.2.1. FurA and their orthologues

Biochemical and genetic studies aimed at discovering the functions of the three members of the family identified in *Anabaena* (*Nostoc*) PCC 7120, namely FurA, FurB and FurC (Hernández, López-Gomollón *et al.*, 2004), showed that FurA, the *all1691* gene product, is the most abundant of the three proteins under standard culture conditions. FurA is a master regulator acting as a hub that connects iron homeostasis, oxidative stress defence and other relevant metabolic pathways (Fillat, 2010). In *Anabaena*, FurA seems both to act as the ferric uptake regulator and to perform the PerR functions. Unlike the other two Fur paralogues, FurA is an essential protein under standard culture conditions (González, Bes, Barja, Peleato, & Fillat, 2010; Hernández, Muro-Pastor *et al.*, 2006). *Anabaena* mutants overexpressing FurA have lower iron content than the WT strain and an iron-deficient phenotype (González *et al.*, 2010; Hernández *et al.*, 2010). Because of the tight connection between iron metabolism and oxidative stress, the fact that the same protein may respond to both signals would allow a more efficient co-ordination between iron uptake and storage and the redox status of the cell. Since the current working model for FurA is based on the repression of target genes using  $\text{Fe}^{2+}$  as co-repressor, a dual-sensing mechanism could rely on iron oxidation produced by oxidative damage that, in turn, would lead to the dissociation of Fur from the DNA, thus allowing the transcription of genes involved in ROS quenching (Fig. 4.3). Single-molecule assays show the tendency of FurA to form trimers and higher aggregates via disulphide bridges in the presence of  $\text{H}_2\text{O}_2$  (Lostao, Peleato, Gomez-Moreno, & Fillat, 2010), suggesting that oxidative injury could irreversibly produce nonfunctional dimers, activating the concerted response to oxidative stress.

Similar to *Anabaena* PCC 7120, the genome of *Synechocystis* PCC 6803 contains three *fur* paralogues. As in the case of *furA*, attempts to inactivate the *sll0567* gene resulted in partially segregated mutants (Kunert, Vinnemeier,

**Table 4.1** Fur orthologues and their gene contexts in different cyanobacterial genera

Cyanobacterial strain	<i>fur</i> orthologues	Gene upstream	Gene downstream
<i>Anabaena</i> PCC 7120	<i>all</i> 1691 ( <i>furA</i> )	<i>sigC</i>	Cell-wall binding protein
	<i>all</i> 2473 ( <i>furB/zur</i> )	<i>asr</i> 2474 (hypothetical protein)	<i>alr</i> 2472 (hypothetical protein)
	<i>alr</i> 0957( <i>furC</i> )	<i>all</i> 0956 (unknown protein)	<i>all</i> 0958 (unknown protein)
<i>Synechocystis</i> PCC 6803	<i>sll</i> 0567 ( <i>furA</i> )	<i>slr</i> 0569 (hypothetical protein)	<i>slr</i> 0594 (hypothetical protein)
	<i>slr</i> 1738 ( <i>perR</i> )	<i>ahpC</i> /sodB	<i>sll</i> 1620 (hypothetical protein)
	<i>sll</i> 1937 ( <i>furB/zur</i> )	Zn <sup>2+</sup> transport protein	<i>sll</i> 1938 (hypothetical protein)
<i>Microcystis aeruginosa</i> NIES-843	MAE 37080 ( <i>furA</i> )	<i>hsp40</i>	<i>sufE</i>
	MAE 57540 ( <i>furB/zur</i> )	<i>purS</i>	<i>accD</i>
	MAE 28250 ( <i>furC</i> )	MAE 28260 (unknown protein)	MAE 28240 (hypothetical protein)
<i>Gloeobacter violaceus</i> PCC 7421	<i>glr</i> 3304( <i>furA</i> )	<i>glr</i> 3303 (unknown protein)	Probable hydrolase
	<i>glr</i> 3733 ( <i>perR</i> )	<i>murD</i>	Rubrerhythrin-like protein
	<i>glr</i> 0783 ( <i>furB/zur</i> )	<i>glr</i> 0782 (hypothetical protein)	<i>phrA</i>
<i>Synechococcus elongatus</i> PCC 7942	<i>Synpcc</i> 7942_0987 ( <i>furA</i> ★)	<i>Synpcc</i> 7942_0988 (hypothetical protein)	Probable glycosyl transferase
	<i>Synpcc</i> 7942_2170 ( <i>furA</i> )	<i>Synpcc</i> 7942_2169 (hypothetical protein)	<i>dpsA</i>
	<i>Synpcc</i> 7942_1648 ( <i>perR</i> )	Rubrerhythrin	<i>Synpcc</i> 7942_1647 (hypothetical protein)
	<i>Synpcc</i> 7942_0817 ( <i>furB/zur</i> )	<i>purS</i>	Diguanylate cyclase/ phosphodiesterase
	<i>Synpcc</i> 7942_1803 ( <i>furC</i> )	<i>Synpcc</i> 7942_1804 (hypothetical protein)	Response regulator CheY-like
<i>Thermosynechococcus elongatus</i> BP-1	<i>tll0048</i> ( <i>furA</i> )	Penicillin-binding protein	<i>tll0047</i> (unknown protein)
	<i>tlr0192</i> ( <i>furB/zur</i> )	<i>tlr0191</i> (hypothetical protein)	<i>tlr0193</i> (hypothetical protein)
	<i>tll0025</i> ( <i>furC</i> )	<i>tll026</i> (hypothetical protein)	<i>tll0024</i> (hypothetical protein)

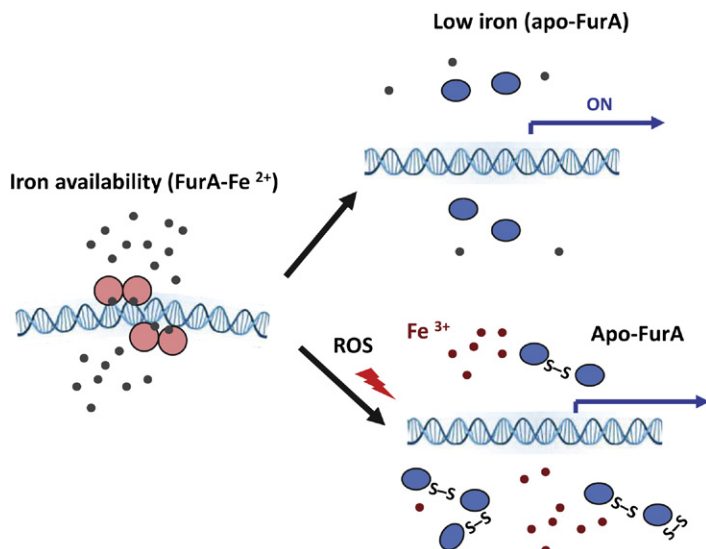
Continued

**Table 4.1** Fur orthologues and their gene contexts in different cyanobacterial genera—cont'd

Cyanobacterial strain	<i>fur</i> orthologues	Gene upstream	Gene downstream
<i>Cyanothece</i> PCC 7425	<i>Cyan7425_1789 (furA)</i>	RNA polymerase, sigma 70 subunit	CAB/ELIP/HLIP superfamily protein
	<i>Cyan7425_1597 (perR)</i>	Peroxiredoxin	Glycosyl transferase
	<i>Cyan7425_4383 (furB/zur)</i>	<i>Cyan7425_4384</i> (hypothetical protein)	<i>Cyan7425_4382</i> (hypothetical protein)
	<i>Cyan7425_1917 (furB/zur)</i>	Periplasmic solute binding protein	<i>Cyan7425_1918</i> (hypothetical protein)
	<i>Cyan7425_2524 (furC)</i>	UbiD family decarboxylase	Protein of unknown function DUF151
<i>Trichodesmium erythraeum</i> IMS101	<i>Tery_1958 (furA*)</i>	Lipoyl synthase	Peptidoglycan binding domain protein
	<i>Tery_3404 (furA)</i>	<i>Tery_3405</i> (hypothetical protein)	<i>Tery_3403</i> (hypothetical protein)
	<i>Tery_1953 (furB/zur)</i>	<i>purS</i>	SPFH domain/band 7 protein
<i>Prochlorococcus marinus</i> MED4	<i>PMM0637 (furA)</i>	Isochorismatase hydrolase	<i>PMM0638</i> (hypothetical protein)
	<i>PMM1030 (furB/zur)</i>	ABC transporter, possibly Mn <sup>2+</sup> transport	ABC transporter, possibly Mn <sup>2+</sup> transport
<i>Anabaena variabilis</i> ATCC 29413	<i>Ava_1165 (furA)</i>	<i>sigC</i>	Peptidoglycan binding domain protein
	<i>Ava_0405 (furB/zur)</i>	<i>purS</i>	SPFH domain/band 7 protein
	<i>Ava_0536 (furC)</i>	Rieske (2Fe-2S) region	Pentapeptide repeat protein
<i>Nostoc punctiforme</i> ATCC 29133	<i>Npun_F0997 (furA)</i>	RpoD family RNA polymerase sigma factor	Peptidoglycan binding domain protein
	<i>Npun_R6581 (furB/zur)</i>	<i>purS</i>	Band 7 protein
	<i>Npun_R5017 (furC)</i>	<i>Npun_F5018</i> (hypothetical protein)	Pentapeptide repeat protein

<i>Arthrospira platensis</i> NYES_39	<i>NYES39_B00070 (furA)</i>	<i>sigC</i>	Peptidoglycan binding domain protein
	<i>NYES39_M02560 (perR)</i>	Putative peroxiredoxin	<i>accA</i>
	<i>NYES39_K04260 (furB/zur)</i>	<i>NYES39_K04270</i> (hypothetical protein)	<i>aspS</i>
<i>Acaryochloris marina</i> MBIC 11017	<i>AM1_2111 (furA★)</i>	<i>rpoD</i>	<i>AM1_2110</i> (unknown protein)
	<i>AM1_F0075 (furA)</i>	<i>AM1_F0076</i> (hypothetical protein)	<i>fur</i>
	<i>AM1_B0142 (furA)</i>	<i>AM1_B0141</i> (hypothetical protein)	<i>AM1_B0143</i> (hypothetical protein)
	<i>AM1_A0268(furA)</i>	<i>AM1_A0267</i> (hypothetical protein)	<i>AM1_A0269</i> (hypothetical protein)
	<i>AM1_1131(furA)</i>	<i>psbE</i>	<i>AM1_1132</i> (hypothetical protein)
	<i>AM1_3714 (perR)</i>	<i>katG</i>	Acetyltransferase
	<i>AM1_3679 (perR)</i>	Peroxiredoxin	<i>AM1_3678</i> (hypothetical protein)
	<i>AM1_F0074 (perR)</i>	<i>AM1_F0073</i> (hypothetical protein)	<i>fur</i>
	<i>AM1_B0144 (perR)</i>	<i>AM1_B0145</i> (hypothetical protein)	<i>AM1_B0143</i> (hypothetical protein)
	<i>AM1_3167 (furB/zur)</i>	<i>AM1_3168</i> (hypothetical protein)	<i>AM1_3165</i> (hypothetical protein)
	<i>AM1_F0079 (furB/zur)</i>	<i>AM1_F0078</i> (hypothetical protein)	<i>AM1_F0081</i> (hypothetical protein)
	<i>AM1_3038 (furB/zur)</i>	<i>AM1_3039</i> (hypothetical protein)	<i>AM1_3037</i> (hypothetical protein)
	<i>AM1_B0139 (furB/zur)</i>	<i>AM1_B0140</i> (hypothetical protein)	<i>AM1_B0138</i> (hypothetical protein)
	<i>AM1_6352 (furC)</i>	<i>AM1_6353</i> (hypothetical protein)	<i>hemeE</i>

*furA★*: Shows the highest homologue with *furA* (*all1691*).



**Figure 4.3** Proposed model for the influence of the intracellular iron concentration and the presence of reactive oxygen species (ROS) in FurA activity. See the colour plate.

Erdmann, & Hagemann, 2003; Nakamura, Kaneko, Miyajima, & Tabata, 1999), whose phenotypes together with the high sequence homology between Sll0567 and FurA (81%) indicates that both *all1691* and *sll0567* genes are orthologues.

### 3.1.2.2. PerR and the oxidative stress response

Inactivation of the *Synechocystis* PCC 6803 *slr1738* gene and functional genomics of the resulting disruption mutant under different stress conditions (Li, Singh, McIntyre, & Sherman, 2004; Singh, Li, & Sherman, 2004) clearly point to this gene as a *perR* orthologue that is involved in the control of the peroxide stress response in this unicellular cyanobacteria. Induction of Slr1738 by H<sub>2</sub>O<sub>2</sub> and the higher tolerance of the  $\Delta$ *slr1738* mutant to peroxide and paraquat consistent with the increased expression of several antioxidant genes also support those results (Houot *et al.*, 2007).

A comparison of differentially regulated genes of the peroxide stimulon in *Synechocystis* PCC 6803 and in the  $\Delta$ *perR* mutant strain allowed identifying a novel type 2 peroxiredoxin gene, namely *sll1621*, as a main PerR target. The PerR regulon (Table 4.2) also includes some genes related to iron homeostasis, such as *idiA* and *mrgA* (Li *et al.*, 2004). Moreover, the exposure to methyl viologen under normal or high light allowed the identification

**Table 4.2** PerR-regulated genes in *Synechocystis* PCC 6803 (Kobayashi et al., 2004; Li et al., 2004)

Gene ID	Synonym	Gene product description
<i>slr1738</i>	<i>perR</i>	Peroxide regulon repressor
<i>sll1621</i>	<i>ahpC</i>	Alkyl hydroperoxide reductase
<i>sll1620</i>		Unknown protein
<i>slr1739</i>	<i>psbW-like</i>	Unknown protein
<i>ssl2667</i>	<i>cnfU</i>	NifU-like C-terminal
<i>sll0621</i>	<i>dsb-like</i>	c-type cytochrome biogenesis
<i>slr0513</i>	<i>idiA</i>	Iron-deficiency-induced protein A homologue
<i>sll0247</i>	<i>isiA</i>	Iron-stress-inducible Chl-binding protein IsiA
<i>sll1135</i>		Unknown protein
<i>sll1483</i>		Fasciclin-like domain
<i>slr1894</i>	<i>mrgA</i>	Metal-regulated gene
<i>slr1204</i>	<i>htrA</i>	HtrA protease
<i>sll1450</i>	<i>nrtA</i>	Nitrate/nitrite transport system substrate-binding protein
<i>sll1688</i>	<i>thrC</i>	Threonine synthase
<i>sll0381</i>		Hypothetical protein
<i>sll0684</i>	<i>pstB</i>	Phosphate transport ATP-binding protein PstB homologue
<i>sll1030</i>	<i>ccmL</i>	Carbon dioxide concentrating mechanism protein CcmL
<i>sll0680</i>	<i>pstS</i>	Phosphate-binding periplasmic protein precursor (PBP)
<i>sll1031</i>	<i>ccmM</i>	Carbon dioxide concentrating mechanism protein CcmM
<i>sll0616</i>	<i>secA</i>	Preprotein translocase SecA subunit
<i>slr0900</i>	<i>moeA</i>	Molybdopterin biosynthesis MoeA protein
<i>sll0927</i>	<i>metX</i>	S-adenosylmethionine synthetase
<i>slr0898</i>	<i>nirA</i>	Ferredoxin-nitrite reductase
<i>slr0364</i>		Hypothetical protein
<i>sll0681</i>	<i>pstC</i>	Phosphate transport system permease protein PstC homologue
<i>sll1032</i>	<i>ccmN</i>	Carbon dioxide concentrating mechanism protein CcmN
<i>sll0385</i>	<i>cbiO</i>	ATP-binding protein of ABC transporter
<i>slr0899</i>	<i>cynS</i>	Cyanate lyase
<i>sll0685</i>		Hypothetical protein
<i>sll1780</i>	<i>ISY203b</i>	Putative transposase
<i>sll1804</i>	<i>rps3</i>	30S ribosomal protein S3
<i>sll1397</i>	<i>ISY100a</i>	Putative transposase
<i>sll1071</i>		Hypothetical protein
<i>slr0628</i>	<i>rps14</i>	30S ribosomal protein S14
<i>slr1616</i>		Unknown protein
<i>sll1771</i>	<i>pphA</i>	Protein serine–threonine phosphatase
<i>slr1931</i>	<i>pilA8</i>	Type 4 pilin-like protein



of PerR-regulated genes involved in nitrogen metabolism (*nrtA* and *nirA*), photosynthesis (*ccmL*, *ccmM*, and *ccmN*) and nutrient transport (*pstB* and *pstC* homologues) among other functional categories (Kobayashi *et al.*, 2004). However, some of these results also showed that many genes responding to oxidative stress are PerR independent, indicating that Slr1738/PerR is not the only regulator of the concerted response to peroxide stress in *Synechocystis* (Li *et al.*, 2004; Kobayashi *et al.*, 2004).

Concerning *Anabaena* PCC 7120, neither FurB nor FurC seem to function as PerR orthologues, pointing to FurA as a dual responsive regulator that participates in the control of iron metabolism and the oxidative stress response. The pathway leading to induction of the peroxide stimulon is not yet well understood in cyanobacteria. Disruption of cellular homeostasis by other environmental stresses lead to enhanced generation of ROS that could act as a cellular signal or be a part of a systemic response. Under adverse conditions, the cyanobacterial transcriptome is reprogrammed to allow an optimal cellular co-ordination (Singh *et al.*, 2010) and PerR has been found among the commonly regulated genes under most perturbations.

### 3.1.2.3. FurB from *Anabaena*: a moonlighting protein?

FurB orthologues are present in almost all cyanobacteria except cyanobacterium UCYNA. Although most cyanobacteria contain a single *furB* gene, *A. marina* MBIC11017 genome harbours four homologues, two of them encoded by a plasmid and some *Cyanothece* strains possess two *furB* homologues. In contrast to FurA proteins, the *furB* orthologues in *Synechocystis* PCC 6803 (*sll1937*) and *Anabaena* PCC 7120 (*all2473*) are dispensable under standard culture conditions (Kunert *et al.*, 2003; Napolitano *et al.*, 2012). In *Synechocystis* PCC 6803, *sll1937/furB* seems not to be involved in the control of the *isiAB* operon (Kunert *et al.*, 2003) and the constitutive expression of the Zn<sup>2+</sup> uptake transporter operon *znuCAB* in the *sll1937*-inactivated mutant strongly suggests that Sll1937 works as a Zur protein in this cyanobacteria (Pakrasi, Ogawa, & Bhattacharrya-Pakrasi, 2004).

In *Anabaena* PCC 7120, it has been suggested that FurB, the product of *all2473*, may protect cells against oxidative stress, most likely by direct protection of the cyanobacterial nucleoid, in a similar way to a Dps protein (López-Gomollón *et al.*, 2009). This hypothesis is based on the fact that expression of the gene is strongly induced under oxidative challenge and its overexpression in *E. coli* increases its tolerance to H<sub>2</sub>O<sub>2</sub> and paraquat. In addition, FurB presents a very high isoelectric point (pI 8.7), similar to histones, and protects DNA *in vitro* from ROS and DNaseI damage.

In a recent study (Napolitano et al., 2012), FurB has been identified as a Zur regulator in *Anabaena* PCC 7120. Unlike most FurA-modulated genes that exhibit two or more iron boxes, specific binding of FurB to DNA occurs in the single 7-1-7 consensus motif (TGATAATNATTATCA). In *Anabaena* PCC 7120, FurB is involved in the control of at least 23 genes, 17 of them organized in six operons. FurB-controlled genes fall into four different categories, namely paralogues of zinc metalloproteins, putative metallochaperones, components of ABC transporters and outer membrane proteins (Table 4.3).

These data indicate that FurB/Zur is a main component, together with SmtB proteins (Huckle, Morby, Turner, & Robinson, 1993; Thelwell, Robinson, & Turner-Cavet, 1998), in the response and adaptation of cyanobacteria to  $\text{Zn}^{2+}$  deficiency and suggests that FurB might be a moonlighting protein, playing a dual role in *Anabaena*.

#### 3.1.2.4. FurC: a potential regulator of regulators

A third Fur paralogue, FurC (Alr0957), has been identified and purified in *Anabaena* PCC 7120 (Hernández, López-Gomollón et al., 2004). The FurC basal expression level is lower than those of FurA and FurB and exhibits a significant gap in homology with those proteins. As other members of this family, FurC is able to dimerize although the dimer is only detected under oxidizing conditions. Our hypothesis is that FurC can form heterodimers through its C-terminus with other Fur family members, modifying their affinity for DNA. Residues involved in C-terminal domain dimerization are quite conserved in FurC, allowing the potential formation of heterodimers. Reverse genetics studies are expected to rule out that FurC can have another role, so far unknown, including target promoters not yet identified.

#### 3.1.3. Deciphering the FurA regulon

The potentially essential role of FurA in *Anabaena* sp. physiology poses the challenge of deciphering its regulon. Comparative global analyses of transcriptomes and proteomes for *fur* deletion mutants and their parental wild-type strains have been traditionally used to characterize Fur regulons in several heterotrophic bacteria (Baichoo, Wang, Ye, & Helmann, 2002; Gao et al., 2008; McHugh et al., 2003; Wan et al., 2004). However, gene knock-out cannot be used to define function when silencing implies death. In such cases, alternative approaches such as overexpression (Olmedo-Verd, Flores, Herrero, & Muro-Pastor, 2005; Wu, Liu, Lee, & Golden, 2004) or selectively regulating gene expression (Callahan & Buikema, 2001; Zhang et al., 2000)

**Table 4.3** FurB/Zur-regulated genes in *Anabaena* PCC 7120 (Napolitano *et al.*, 2012)

Functional category	Gene ID	Synonym	Gene product description
ABC transport systems	<i>all0833</i>	<i>znuA</i>	Periplasmic solute binding protein
	<i>all0832</i>	<i>znuB</i>	ABC transporter, ATP-binding protein
	<i>all0830</i>	<i>znuC</i>	ATP-transporter permease protein
	<i>alr3243</i>		ABC transporter, periplasmic-binding protein
	<i>alr4031</i>		ABC transporter, periplasmic-binding protein (COG0614)
Outer membrane proteins	<i>alr3242</i>		TonB-dependent receptor (COG1629)
	<i>alr4028-4029</i>		TonB-dependent transporter (outer membrane)
	<i>all3515</i>		Putative outer membrane protein
Paralogues of zinc metalloproteins	<i>all7621</i>	<i>aztR</i>	ArsR/SmtB family transcriptional regulator
	<i>all47121</i>	<i>folE</i>	GTP-cyclohydrolase
	<i>all4723</i>	<i>thrS2</i>	Threonyl-tRNA synthetase
	<i>all4725</i>	<i>hemE</i>	Porphobilinogen synthase
Operons containing putative metallochaperones	<i>alr1197</i>		CobW-C superfamily
	<i>all1198</i>		Metallophosphoesterase COG0622
	<i>alr1199</i>		Metallo-dependent phosphatase
	<i>all1751</i>		Putative metallochaperone COG0523
	<i>all1750</i>		WD40 repeat-containing protein
Glycosyl transferases	<i>alr2866</i>		Glycosyl transferase, family 2
Others	<i>alr3495</i>		Uncharacterized conserved protein COG1262
	<i>all1474</i>		CRISPR-associated RAMP protein, SSO1426 family COG1337
	<i>alr4030</i>		Putative ferredoxin (thiorredoxin fold) COG 3411
	<i>all4722</i>		P-loop GTPase (COG0523 family)
	<i>all4724</i>		Putative FAD-dependent oxidoreductase

often overcome this limitation and can be used to discern functions, unravel regulation mechanisms or identify direct targets within regulatory networks.

The first studies discerning the FurA regulon showed that this metallorepressor specifically bound in vitro to A/T-rich sequences of its own promoter, while the absence of both divalent metal ions and/or reducing conditions as well as the presence of haeme severely impaired its affinity for DNA (Hernández, López-Gomollón et al., 2006; Hernández, Peleato et al., 2004). Surprisingly, the expression of FurA appeared strongly enhanced in proheterocysts and mature heterocysts, and this upregulation seemed to be mediated by NtcA, a master regulator of nitrogen metabolism that triggers heterocyst differentiation and nitrogen fixation in diazotrophic cyanobacteria (López-Gomollón, Hernández, Wolk, Peleato, & Fillat, 2007). These findings represented the first evidence of FurA involvement in nitrogen metabolism and led to further definition of a cross-talk between FurA and NtcA, identifying overlapping genes in both regulons (López-Gomollón, Hernández, Pellicer et al., 2007).

More recently, overexpression has been successfully used as an alternative method to gain new insights into the FurA regulatory function. Overexpression of FurA in *Anabaena* PCC 7120 induced changes in the transcriptional pattern of a variety of genes, leading to alterations in photoautotrophic growth, filament integrity, cell morphology, ultrastructure, photosynthetic function and defence against oxidative stress (González et al., 2010). Although some of the effects observed under a FurA overexpression phenotype could result from an aberrant response unrelated to the normal function of the protein, the combination of phenotypic studies with both transcriptional and proteomic profile variations in conjunction with FurA–DNA interaction analyses allowed more than 20 new direct targets of this transcriptional regulator to be identified (González et al., 2010, 2011).

Of the three different Fur homologues described in *Anabaena* sp. (Hernández, López-Gomollón et al., 2004), FurA is the master regulator of iron homeostasis, controlling the expression of iron uptake and the storage machinery in response to iron availability (González A. et al., 2012). However, the same protein appears to have a direct regulatory role in the transcription of several genes involved in oxidative stress defences and redox signalling, modulating the expression of at least two peroxiredoxins (González et al., 2011), thioredoxin (López-Gomollón, Hernández, Pellicer et al., 2007), thioredoxin reductase (González et al., 2011) and DpsA-homologues (Hernández, Pellicer, Huang, Peleato, & Ft, 2007). Since DNA-binding activity of FurA is critically dependent on reducing conditions

(Hernández, López-Gomollón *et al.*, 2006) and its expression is slightly induced under oxidative stress (López-Gomollón *et al.*, 2009), FurA could also act as an oxidative stress-responsive regulator, similar to other members of the Fur family like PerR of gram-positive bacteria (Herbig & Helmann, 2001; Ricci, Janulczyk, & Bjorck, 2002).

Overall, the variety of FurA-regulated genes described so far (Table 4.4), including siderophore outer membrane transporters, bacterial actins, photosystem II reaction centre proteins, CO<sub>2</sub> concentrating mechanism proteins and peroxiredoxins, provides evidence that FurA functions as a global transcriptional regulator in *Anabaena* sp., supporting its role in major cyanobacterial processes.

#### **3.1.4. Genetic regulation of cyanobacterial Fur proteins**

Most Fur proteins studied to date show moderate autoregulation. FurA from *Anabaena* binds to its own promoter with an estimated K<sub>d</sub> of  $0.49 \pm 1$  nM, the presence of Mn<sup>2+</sup> and a reducing environment being the optimal conditions for in vitro FurA–P<sub>*furA*</sub> interaction (Hernández, López-Gomollón *et al.*, 2006). In vivo assays aimed at understanding the relationship between the regulation and the functions of this master protein unveil a rather complex model. A slight increase in the expression of FurA has been detected under iron limitation (Hernández *et al.*, 2002). Since iron deprivation leads to oxidative stress, the increase in FurA expression could be explained as a response to the rise in the level of ROS detected in the cell under iron deficiency (Latifi, Jeanjean, Lemeille, Havaux, & Zhang, 2005). This hypothesis is consistent with the observation that oxidants trigger *furA* transcription (López-Gomollón *et al.*, 2009). This increase in FurA expression might be used by the cyanobacteria to downregulate iron uptake in order to arrest catalysis of the Fenton reaction. Alternatively, a nonregulatory role directly involving FurA in ROS quenching has been proposed. Work intended to establish a plausible mechanism of FurA acting as a redox protein based on its two CXXC redox motifs is underway (Botello-Morte *et al.*, our unpublished results).

Northern blot analysis of *furA* under nitrogen stepdown in *Anabaena* PCC 7120 and the *ntcA* deletion mutant evidenced that the nitrogen status modulates FurA expression and that NtcA is involved in this process (López-Gomollón, Hernández, Wolk *et al.*, 2007). In order to know whether the increase in *furA* transcription was a general response in the cyanobacterial filament, constructs of the promoters from each *fur* paralogue leading the expression of GFP were used to identify a strong induction of *furA* in heterocysts.

**Table 4.4.** FurA regulated genes in *Anabaena* sp. PCC 7120

Functional category	Gene ID <sup>a</sup>	Gene product description	Reference
Iron metabolism	<i>schT</i>	Siderophore outer membrane transporter	González et al., 2010
	<i>all1101</i>	Siderophore outer membrane transporter	González et al., 2012
	<i>all2610</i>	Siderophore outer membrane transporter	González et al., 2012
	<i>alr3242</i>	TonB-dependent heme receptor	González et al., 2012
	<i>alr3240</i>	Ferrichrome ABC transporter	González et al., 2012
	<i>all0389</i>	Iron (III) ABC transporter	González et al., 2012
	<i>all2618</i>	Iron (III) dicitrate-binding periplasmic protein	González et al., 2012
	<i>all2641–all2649</i>	Peptide synthetases / polyketide synthases gene cluster	González et al., 2012
Oxidative stress defenses and redox regulation	<i>all4145</i>	Ferritin, Dps-family protein	González et al., 2012
	<i>dpsA</i>	Nutrient-stress induced DNA binding protein	Hernández et al., 2007
	<i>gor</i>	Glutathione reductase	López-Gomollón et al., 2007a
	<i>trxA</i>	Thioredoxin	López-Gomollón et al., 2007a
	<i>trxB</i>	Thioredoxin-reductase	González et al., 2011
	<i>all1541</i>	Peroxiredoxin 2 family protein/glutaredoxin	González et al., 2011
Photosynthesis and respiration	<i>alr4641</i>	Peroxiredoxin	González et al., 2011
	<i>isiA</i>	Photosystem II chlorophyll $\alpha$ -binding protein	Leonhardt et al., 1994
	<i>isiB</i>	Flavodoxin	Bes at al., 2001
	<i>rbcL</i>	Ribulose 1,5-bisphosphate carboxylase/oxygenase large subunit	López-Gomollón et al., 2007a
	<i>psaL</i>	Photosystem I subunit XI	López-Gomollón et al., 2007a
	<i>psbZ</i>	Photosystem II 11 kDa protein	López-Gomollón et al., 2007a
	<i>coxA</i>	Cytochrome C oxidase subunit I	López-Gomollón et al., 2007a
	<i>coxB</i>	Cytochrome C oxidase subunit II	López-Gomollón et al., 2007a
	<i>ndhF</i>	NADH dehydrogenase subunit 5	López-Gomollón et al., 2007a
	<i>petH</i>	Ferredoxin-NADP(+) reductase	López-Gomollón et al., 2007a
	<i>prk</i>	Phosphoribulokinase	López-Gomollón et al., 2007a
	<i>psbA</i>	Photosystem II reaction center protein D1	González et al., 2010
	<i>ccmM</i>	CO <sub>2</sub> concentrating mechanism protein	González et al., 2011

*Continued*

**Table 4.4.** FurA regulated genes in *Anabaena* sp. PCC 7120—cont'd

Functional category	Gene ID <sup>a</sup>	Gene product description	Reference
Nitrogen metabo- lism	<i>ntcA</i>	Master transcriptional regulator of nitrogen metabolism	López-Gomollón et al., 2007a
	<i>glnA</i>	Glutamate-ammonia ligase	López-Gomollón et al., 2007a
	<i>gltS</i>	Ferredoxin-glutamate synthase	López-Gomollón et al., 2007a
	<i>nifH</i>	Nitrogenase iron protein	López-Gomollón et al., 2007a
	<i>abp1</i>	DNA binding protein	González et al., 2011
Tetrapyrrole bio- synthesis	<i>hemB</i>	Porphobilinogen synthase	González et al., 2012
	<i>hemC</i>	Porphobilinogen deaminase	González et al., 2012
	<i>hemK</i>	Protoporphyrinogen IX oxidase	González et al., 2012
	<i>hemH</i>	Ferrochelatase	González et al., 2012
	<i>ho1</i>	Heme-oxygenase 1	González et al., 2012
Others	<i>furA</i>	Ferric uptake regulator	Bes at al., 2001
	<i>furB</i>	Ferric uptake regulator	Hernández et al., 2004
	<i>furC</i>	Ferric uptake regulator	Hernández et al., 2004
	<i>sigC</i>	RNA polymerase sigma-subunit	López-Gomollón et al., 2007a
	<i>hanA</i>	DNA binding protein HU	López-Gomollón et al., 2007a
	<i>α-furA<sup>b</sup></i>	Antisense RNA	López-Gomollón et al., 2007b
	<i>mreBCD</i>	Operon encoding bacterial actins	González et al., 2010
	<i>all3556</i>	Succinate-semialdehyde dehydrogenase	González et al., 2011
	<i>tldD</i>	Putative modulator of DNA gyrase	González et al., 2011
	<i>pmbA</i>	Putative modulator of DNA gyrase	González et al., 2011
	<i>orrA</i>	Two-component response regulator	González et al., 2011
	<i>thiC</i>	Thiamin biosynthesis protein	González et al., 2011

<sup>a</sup>Gene identification according to the cyanobacteria genome database CyanoBase (<http://genome.kazusa.or.jp/cyanobase>)

<sup>b</sup>Gene identification according to Hernández et al, 2006b

The identification of an antisense RNA that covers the complete *furA* gene and is cotranscribed with the cell wall-binding protein Alr1690 adds another mechanism for dynamic, fine-tuning *furA* regulation at the post-transcriptional stage (Hernández, Muro-Pastor et al., 2006). Insertional inactivation of the *alr1690- $\alpha$ -furA* dicistronic message produces smaller cells exhibiting a 2.5-fold increase in FurA expression and 62% of iron content with respect to *Anabaena* WT.  $\Delta$ *alr1690- $\alpha$ -furA* cells display a reduced number of contorted thylakoids, as well as alterations in the photosynthetic apparatus, leading to lower photosynthetic performance indexes (Hernández et al., 2010). These results indicate that the expression of the *alr1690- $\alpha$ -furA* message is required for the maintenance of a proper thylakoid arrangement, efficient regulation of iron uptake and optimal yield of the photosynthetic machinery.

The occurrence of anti-*fur* RNAs has been found in other cyanobacterial strains, namely *Microcystis aeruginosa* PCC 7806 and *Synechocystis* PCC 6803, showing rather different gene contexts between them (Sevilla et al., 2011). In the case of *Microcystis*, the anti-*fur* RNA spans the whole *Mafur* CDS and part of the flanking *dnaJ* and *sufE* sequences, while Sy $\alpha$ -*fur* RNA covers only part of the coding sequence of the *fur* orthologue *sll0567*. It has been reported that in heterotrophic bacteria, Fur can indirectly activate several genes by repressing trans-acting, small antisense RNAs, such as RyhB in *E. coli* or the functional homologues PrrF in *Pseudomonas* and NrrF in *Neisseria* (Metruccio et al., 2009; Wilderman et al., 2004). However,  $\alpha$ -*furA* is the first antisense RNA reported to modulate a Fur protein. The question of whether cyanobacterial Fur proteins can also repress small nc-RNAs will require further work addressing functional transcriptomics of the many encoded regulatory RNAs found in cyanobacteria (Georg & Hess, 2011).

At the post-translational level, the DNA-binding ability of FurA is enhanced by the presence of FurC in contrast to the inhibition observed when FurA is complexed with haeme (Hernández, López-Gomollón et al., 2004; Hernández, Peleato et al 2004). The estimated  $K_d = 0.4 \pm 0.1 \mu\text{M}$  for the FurA–haeme interaction strongly suggests that the binding takes place in vivo as a regulatory mechanism, likely acting as a haeme-sensor protein (Pellicer et al., 2012). In summary, the regulatory model for FurA from *Anabaena* can be presented as a complex balance of several signals that influence the final concentration of this protein along the three steps of the genetic flow of information.

Concerning other FurA paralogues, in vitro assays indicate that FurB and FurC might be regulated by FurA since the latter binds to their promoters.



RT-PCR assays confirm the influence of FurA on *furB* transcription that clearly decreases in the FurA overexpressing mutant (González *et al.*, 2010). The influence of several nutritional and environmental factors on *furB* and *furC* expression was investigated by RT-PCR and using GFP constructs driven by their promoters (López-Gomollón *et al.*, 2009). Among the conditions tested, neither osmotic stress induced by sucrose nor salt stress affected by FurB or FurC expression. However, oxidative challenge induced by H<sub>2</sub>O<sub>2</sub> enhanced the expression of both genes.

Similar to FurA, FurB exhibits the CP haeme-regulatory motif, and binding to this cofactor impairs its interaction with DNA. In vitro assays show that FurB binds to its own promoter (Hernández, López-Gomollón *et al.*, 2004). This interaction is stronger in the absence of divalent metals and it is destroyed in the presence of Zn<sup>2+</sup>. Reducing conditions managed by the presence of DTT positively affected FurB–DNA interaction. Further work involving in vivo studies should be done in order to address the role of Zn<sup>2+</sup> in FurB autoregulation.

### **3.1.5. Metabolic and regulatory networks involving Fur proteins**

Among the *fur* paralogues identified in cyanobacteria, *furA* and their orthologues seem to be the most important for the cell since all attempts to fully inactivate the *furA* genes from cyanobacteria have been unsuccessful under standard growth conditions (Ghassemian & Straus, 1996; Hernández, Muro-Pastor *et al.*, 2006; Kunert *et al.*, 2003; Michel, Pistorius, & Golden, 2001). Therefore, it is not surprising that FurA seems to be involved directly or indirectly in the modulation of genes participating in several metabolic pathways, including nitrogen metabolism, transcription, photosynthesis and respiration and, of course, iron uptake and oxidative stress, among others.

#### **3.1.5.1. Iron homeostasis and the oxidative stress response**

Cyanobacterial iron homeostasis is mainly maintained by FurA orthologues (Ghassemian & Straus, 1996; González *et al.*, 2010; Houot *et al.*, 2007; Kunert *et al.*, 2003; Straus, 1994). The close relationship between iron homeostasis and oxidative stress has been extensively investigated (Cornelis, Wei, Andrews, & Vinckx, 2011; Faulkner & Helmann, 2011; Latifi *et al.*, 2005; Latifi, Ruiz, & Zhang, 2009; Shcolnick, Summerfield, Reyman, Sherman, & Keren, 2009). This connection is even tighter in cyanobacteria, whose need for iron is about 10 times greater than that of heterotrophic bacteria, and whose photosynthetic and respiratory electron transport chains are particularly sensitive to spare ROS generated by the Mehler reaction

(Shcolnick & Keren, 2006). In fact, several members of the FurA regulon (Table 4.4) participate in oxidative stress defence, while some Slr1738 targets in *Synechocystis*, such as *isiA*, *idiA* and *mrgA* are metal-regulated genes (Li et al., 2004). Previous research on the role of IdiA and MrgA show that these proteins play a pivotal role in the coordination of iron homeostasis and the oxidative stress response (Michel & Pistorius, 2004). Moreover, phenotypic analysis of *Synechococcus* mutants lacking IscA and SufA also point to these proteins as important players in the concerted modulation of iron homeostasis and the sensing of redox stress, expanding cyanobacterial strategies to deal with adverse conditions (Balasubramanian, Shen, Bryant, & Golbeck, 2006). Noticeable among these is the response of *Synechocystis* to Cd stress consisting of an integrated reprogramming of the metabolism under the control of the Fur member Slr1738 (PerR) (Houot et al., 2007). Under Cd stress, metal homeostasis (especially Fe and Zn) and high-light tolerance are disturbed, as well as the functionality of the SUF machinery involved in the synthesis and repair of iron–sulphur centres. Slr1738 targets, such as the *isiAB* operon, are induced by Cd and H<sub>2</sub>O<sub>2</sub> and, noteworthy, the addition of iron in the medium increases the cell tolerance to both challenges. Transcriptomic and spectroscopic analyses also indicate that cyanobacteria challenged with H<sub>2</sub>O<sub>2</sub> or Cd use different strategies for supplying Fe atoms to Fe-requiring metalloenzymes and the SUF machinery. Since Cd regulates the Zn-controlled genes *znuA* and *ziaA*, these authors suggest that this pollutant might be transported via Zn-transport systems. Considering that *znuA* has been identified as a member of the FurB/Zur regulon in *Anabaena* (Napolitano et al., 2012), these results suggest that Slr1738 (PerR) and the Zur orthologue Sll1937 might operate in *Synechocystis* as common elements of a regulatory network controlling the stress (likely oxidative stress) generated by Cd. In addition, the control of *ziaA* by SmtB proteins (Fig. 4.6) highlights a potential functional interaction between Fur and SmtB regulators.

### 3.1.5.2. Role of FurA in the modulation of nitrogen metabolism

The iron pool in nitrogen-fixing cyanobacteria must fulfil the nitrogenase complex requirements. This metalloenzyme contains three different types of Fe–S clusters (Burgess & Lowe, 1996), including the iron–molybdenum cofactor (FeMo–co) at the active site, which contains seven atoms of iron. As expected, nitrogen fixation in *Anabaena* decreases under low iron conditions (Sandmann, Peleato, Fillat, Lázaro, & Gómez-Moreno, 1990). At the molecular level, transcription of the *nifHDK* operon, encoding nitrogenase,

and excision of the 11 kb DNA fragment required for its activation take place in iron-starved *Anabaena*, even though cells grew in the presence of combined nitrogen (Razquin, Schmitz, Fillat, Peleato, & Bohme, 1994). Besides, several iron-responsive genes in cyanobacteria, such as *nblA*, *petH*, *pkn41* and *pkn42*, among others, are also modulated by NtcA (Cheng *et al.*, 2006; Luque, Zabulon, Contreras, & Houmard, 2001; Napolitano *et al.*, 2012; Valladares, Muro-Pastor, Fillat, Herrero, & Flores, 1999), the global regulator of nitrogen control. Cross-talk between FurA and NtcA produces a significant overlapping between the regulatory networks controlled by those regulators, involving genes that belong to different functional categories (López-Gomollón, Hernández, Pellicer *et al.*, 2007). All these results provide strong evidence for the link between iron and nitrogen metabolism in cyanobacteria that will also be affected by the redox status of the cell.

#### 3.1.5.3. FurA and carbon metabolism

Carbon fixation in cyanobacteria relies on a proper assembly of holoproteins associated with photosystems, essential for the production of enough reducing power and ATP needed for a good photosynthetic performance. In *E. coli*, the cyclic AMP receptor protein (CRP) regulates expression of the carbon regulon in response to carbon availability (Zhang *et al.*, 2005). In cyanobacteria, the CRP regulons are highly diversified and CRPs have been lost in some lineages (Xu & Su, 2009). However, in the strains where this regulator has been preserved, though CRPs seem to regulate different sets of genes, they are always involved in the modulation of photosynthetic pathways. Several genes encoding components of PSI and PSII from *Anabaena* PCC 7120, as well as *ccmK*, coding for a CO<sub>2</sub> concentrating mechanism protein, belong to the FurA regulon (González *et al.*, 2010, 2011). As has been reported for *E. coli*, defining a potential functional interaction between FurA and Crp remains an interesting problem that will lead to a better understanding of how cyanobacteria allow integration of signals for iron and carbon availability.

#### 3.1.5.4. Modulation of cyanotoxicity

Certain cyanobacterial species can produce a broad range of bioactive secondary metabolites potentially toxic to eukaryotic organisms, called cyanotoxins (Carmichael *et al.*, 2001). Similar to many bacterial toxins, some cyanotoxins are products of modular peptide synthetases and polyketide synthases, as is the case of microcystins, nodularins, cylindrospermopsins or anatoxins-a. The cyclic heptapeptide microcystin is the most commonly

found and one of the most hazardous classes of cyanotoxin. Microcystins are synthesized in a mixed polyketide synthase/nonribosomal peptide synthetase system called microcystin synthetase. The microcystin synthetase complex in *M. aeruginosa* PCC 7806 is encoded by the *mcy* operon (Tillett et al., 2000). FurA from *M. aeruginosa* recognizes and binds *mcy* promoter regions (Martin-Luna et al., 2006), suggesting a transcriptional control by this global regulator. Moreover, iron deficiency induces *mcyD* expression, correlating with higher levels of microcystin in cells (Sevilla et al., 2008). Fur and NtcA, the global nitrate regulator, balance iron, carbon and nitrogen metabolism resulting in a fine control of the expression of the microcystin gene cluster (Kuniyoshi et al., 2011).

In heterotrophic bacteria, Fur regulates peptide synthetase systems involved in the synthesis of virulence factors, such as enterobactin and vibriobactin, among others (Crosa & Walsh, 2002). These nonribosomal peptide synthetases are similar to the enzymes involved in the synthesis of microcystins. Many of these toxins are siderophores, and even though the ecostrategy or physiological meaning of microcystin production is unknown, several observations suggest a link between microcystin production and iron metabolism. During iron depletion, toxic strains of *Microcystis* maintained cell vitality much longer than nontoxic strains (Lyck, Gjølme, & Utkilen, 1996). Moreover, the rate of iron uptake in toxic strains was higher than nontoxic strains (Utkilen & Gjølme, 1995). Comparison between the iron-stress response in toxic and nontoxic strains of *M. aeruginosa* reveals that the adaptation of *Microcystis* to iron stress is highly dynamic and strain specific (Alexova et al., 2011). The ability to produce microcystin seems to give an advantage to toxic cyanobacteria in the early stages of exposure to severe iron stress and may protect the cell from reactive oxygen species-induced damage. All those observations strongly support that microcystin production may be a FurA-controlled physiological response to iron deficiency.

In summary, these connections strongly suggest that Fur proteins play a central role in the adaptation of cyanobacteria to different environmental and nutritional stresses.

### 3.2. Regulation of Iron–Sulphur Cluster Assembly

Iron–sulphur clusters display versatile functions including stabilization of protein structure, gene regulation, environmental sensing and radical generation (Johnson, 1998). Because of their sensitivity to cellular redox status, iron–sulphur clusters are considered as molecular switches for gene regulation at both the transcriptional and translational levels (Kiley & Beinert,

2003). In cyanobacteria, a variety of enzymes crucial for the organization of fully functional photosystems contain these clusters. Such iron–sulphur proteins also include some components of the respiratory electron transport complexes and enzymes involved in the central metabolism.

### **3.2.1. The iron sulphurcluster (*isc*) system**

Cyanobacteria contain two main iron–sulphur assembly systems denoted as *suf* (sulphur utilization factor) and *isc* (iron sulphur cluster) (Takahashi & Tokumoto, 2002; Wollenberg, Berndt, Bill, Schwenn, & Seidler, 2003). Biosynthesis and assembly of iron–sulphur proteins is a highly regulated process. In *E. coli*, the *isc* operon is under the transcriptional control of the IscR repressor, which is encoded as part of the *iscRSUA* locus and auto-regulates its own expression as well as that of *iscSUA* (Schwartz, Djaman, Imlay, & Kiley, 2000). IscR is a member of the Rrf2 family (PF02082) of transcriptional regulators and contains a winged HTH DNA-binding domain. The active form of IscR presents an unstable [2Fe–2S] cluster that is coordinated by three conserved cysteines and a glutamic residue. Under unfavourable conditions, IscR loses its cluster and becomes inactive in the apo form, allowing the full expression of the *isc* machinery. Thus, the regulatory role of IscR relies upon its iron–sulphur cluster and senses redox changes in the cell for the optimal assembly of Fe–S clusters. Cyanobacterial *isc* genes are scattered throughout the genome, and some are present in multiple copies. IscR homologues identified in cyanobacterial genomes display high sequence similarity at the N terminus in the helix–loop–helix, DNA-binding region. However, the homology at the C-terminus is relatively low. Cyanobacterial IscR homologues lack the 16–17 amino acids present at the C-terminus in IscR proteins from other microorganisms. Furthermore, the cysteine residues conserved in heterotrophic bacteria are missing in cyanobacterial IscR homologues, making the presence of an iron–sulphur cluster unlikely. Therefore, it has been proposed that the cyanobacterial IscR homologues might sense changes in iron status via interactions with other sensor proteins containing iron–sulphur clusters, such as IscA and SufA (Wu, 2008).

### **3.2.2. The sulphur utilization factor (*suf*) system**

The *suf* system in cyanobacteria is considerably more important than the *isc* system. Results from reverse genetics studies performed on *Synechococcus* PCC 7002 indicate that many genes belonging to the *suf* regulon are essential in cyanobacteria (Wang *et al.*, 2004). Furthermore, the *suf* system, but not

the *isc* system, is found in the chloroplasts of higher plants, suggesting that photosynthetic organisms may rely primarily on the *suf* system for assembling iron–sulphur clusters for electron transfer cofactors (Balasubramanian et al., 2006). Most Suf proteins are encoded by the *sufBCDS* operon that is highly conserved in cyanobacterial genomes and negatively regulated by the *sufR* gene encoded by the complementary strand (Wang et al., 2004), except in *Prochlorococcus* spp. In addition of SufR regulation, it has been reported that the activation of the *suf* promoters by a shift to high light conditions (Seki et al., 2006).

SufR belongs to the DeoR family of helix–loop–helix proteins that contain an N-terminal DNA-binding domain and four highly conserved cysteine residues near the C-terminus. Active SufR is also an iron–sulphur protein whose binding affinity depends on the presence and redox state of its  $[4\text{Fe}-4\text{S}^{2+1+}]$  clusters (Shen et al., 2007). However, SufR does not present similar structural or function-related motifs to other redox-sensing regulators such as FNR, SoxR and IscR that also possess Fe–S clusters as sensors. Footprinting and biochemical assays show that both apo and holo-SufR exist as dimers and the active form binds to two distinct sequences with different affinities. The fact that the operator sequences contain two perfect inverted repeats (CAAC- $\text{N}_6$ -GTTG and TAAAACAAC- $\text{N}_6$ -GTTGTTTAA) separated by 26 bp and the finding of SufR tetramers has led to propose that DNA bending might be involved in SufR regulation (Shen et al., 2007).

Furthermore, reverse genetics analysis of *Synechococcus* PCC 7002 highlighted the role of SufA and IscA in the modulation of Fe–S cluster homeostasis (Balasubramanian et al., 2006). These studies also showed that Nfu is essential for the Fe–S scaffold in cyanobacteria. Noticeably, Nfu is homologous to the C-terminus of NifU, a key protein for assembly of the Fe/S cluster in *Azotobacter vinelandii* (Fu, Jack, Morgan, Dean, & Johnson, 1994).

### 3.3. Manganese Homeostasis in Cyanobacteria: the ManR and RfrA Regulators

Manganese is particularly important in oxygenic photosynthetic organisms, playing a critical role in forming a cluster of four atoms on the donor side of photosystem II (PSII), which participates in catalysing the water-splitting reaction (Barber, 2008b). The assembly of  $\text{Mn}^{2+}$  ions to form the catalytically active  $\text{Mn}_4\text{-Ca}$  cluster of the oxygen-evolving complex of the PSII reaction centre is a light-driven process termed photoactivation, which occurs during *de novo* formation of PSII as well as during the frequent repair of PSII in response to photoinhibition (Aro et al., 2005; Barber, 2008a).

In *Synechocystis* PCC 6803,  $\text{Mn}^{2+}$  limitation induces changes in the activity and organization of both photosystems, resulting in a reduction of photochemical activity of PSII as is made evident by lower oxygen evolution rates, lower maximal photosynthesis yield of PSII values, and faster plastoquinone reoxidation rates. On the other hand,  $\text{Mn}^{2+}$  deficit leads to loss of PSI activity as a result of loss of PSI core proteins and  $\text{Mn}^{2+}$  limitation-dependent dissociation of PSI trimers into monomers (Salomon & Keren, 2011). Thus, since  $\text{Mn}^{2+}$  is essential to the function of PSII, and even the state of cellular  $\text{Mn}^{2+}$  availability influences the rate of photochemical activities of both photosystems, there is clearly an intricate genetic network for controlling  $\text{Mn}^{2+}$  homeostasis in cyanobacteria (Chandler, Bartsevich, & Pakrasi, 2003; Ogawa *et al.*, 2002; Yamaguchi *et al.*, 2002).

$\text{Mn}^{2+}$  is accumulated in high concentrations in the cytoplasm of prokaryotes by high-affinity uptake systems. In *Synechocystis* PCC 6803,  $\text{Mn}^{2+}$  acquisition takes place through several transport systems. The best known is MntABC, an ABC-type permease that mediates high-affinity transport under starvation conditions (Bartsevich & Pakrasi, 1995). A second high-affinity transporter acting under Mn-sufficient conditions and a low-affinity transporter indirectly observed by transport kinetics have been reported, but they remain to be characterized (Bartsevich & Pakrasi, 1996).  $\text{Mn}^{2+}$  uptake appears to be dependent on active photosynthesis, leading to accumulation in the cyanobacterial envelope layer. The  $\text{Mn}^{2+}$  outer membrane pool is used as a reservoir for intracellular  $\text{Mn}^{2+}$ , which is kept constant at approximately  $10^6$  atoms per cell of which a large fraction is associated with PSII (Keren, Kidd, Penner-Hahn, & Pakrasi, 2002; Salomon & Keren, 2011).

Transcription of the *mntABC* operon in *Synechocystis* sp. occurs under  $\text{Mn}^{2+}$  starvation conditions (nM levels of  $\text{Mn}^{2+}$ ), but not in a Mn-sufficient environment ( $\mu\text{M}$   $\text{Mn}^{2+}$ ). Such an inducible high-affinity  $\text{Mn}^{2+}$ -transport mechanism is controlled via a two-component signal transduction pathway that negatively regulates the expression of the *mntABC* operon (Ogawa *et al.*, 2002; Yamaguchi *et al.*, 2002). This two-component system, also described in *Anabaena* PCC 7120 (Huang & Wu, 2004a, 2004b), includes a membrane-bound histidine kinase, ManS, which senses the extracellular concentration of  $\text{Mn}^{2+}$  ions and activates a transcriptional response regulator, ManR, which specifically binds to the promoter region of *mntABC* to repress the expression of the ABC-type transporter encoded by this operon. Under  $\text{Mn}^{2+}$  starvation conditions, ManS does not generate a signal, resulting in inactivation of ManR and subsequent expression of the *mntABC* operon.



ManS contains a histidine kinase domain in the C-terminal region that includes a phosphorylatable His residue, and two membrane-spanning domains in the N-terminal region. It has been proposed that the region between these two membrane-spanning domains is located in the periplasmic space and perceives the extracellular concentration of  $Mn^{2+}$  ions by providing ligands to  $Mn^{2+}$  (Yamaguchi et al., 2002). The transcriptional response regulator ManR belongs to the OmpR/PhoB subfamily (Martinez-Hackert & Stock, 1997) and contains two functional domains, an N-terminal phosphorylation domain, which includes a phosphorylatable Asp residue that receives the signal of ManS, and a C-terminal DNA-binding domain. Punctual mutation of either the His-205 residue of the *manS* gene or the Asp-52 residue of the *manR* gene induces the expression of the *mntABC* operon, suggesting that both residues are essential for the transduction of  $Mn^{2+}$  signals. Even though unphosphorylated ManR is bound to the promoter regions of *mntABC* in vitro, transcription seems to be repressed only by its phosphorylated form (Yamaguchi et al., 2002).

In *Anabaena* PCC 7120, the ManS/ManR two-component system modulates the expression of genes *all3575*, *all3574* and *alr3576* that encode the homologous proteins of MntABC from *Synechocystis* PCC 6803 (Huang & Wu, 2004b). Besides, this two component  $Mn^{2+}$ -sensing system controls the expression of the natural resistance-associated macrophage protein (Nramp) homologous MntH (Huang & Wu, 2004a), another type of bacterial  $Mn^{2+}$  transporter that is widespread throughout diverse groups of eubacteria (Jakubovics & Jenkinson, 2001).

The transcriptional metalloregressor ManR is specifically bound to the *Anabaena* sp. *mntH* promoter through a DNA sequence of 19 bp composed of two direct repeats in the form of (T/A)ATGA(G/A)A(A/G) separated by 3 bp, which appears highly conserved in the promoter regions of genes encoding MntABC and MntH homologues of several cyanobacteria (Huang & Wu, 2004a). Since this two direct repeats arrangement is typical of DNA recognition motifs from OmpR/PhoB response regulators (Okamura, Hanaoka, Nagadoi, Makino, & Nishimura, 2000), the conservative consensus sequence (T/A)ATGA(G/A)A(A/G) recognized by ManR appears as a novel regulatory DNA motif in cyanobacteria. The binding of ManR to its target promoters occurs through the C-terminal HTH domain, which contains at least three highly conservative amino acids residues among the OmpR/PhoB subfamily regulators that are essential for DNA-binding activity (Huang, Wu, Li, & Liu, 2006). In vitro analyses suggest that two ManR molecules cooperatively bind to the DNA recognition



sequences at the same time, and this cooperativity appears to be mediated by protein-induced DNA deformation since no protein–protein intermolecular interactions occurred between ManR monomers *in vitro* (Huang & Wu, 2005). Although MntH transporters of  $Mn^{2+}$  have been described in several bacterial groups, the regulation of its expression by the two-component signal transduction system ManS/ManR has only been found in cyanobacteria (Huang & Wu, 2004a).

Another Mn-uptake regulation mechanism described in *Synechocystis* PCC 6803, different from the more thoroughly characterized ManS/ManR two-component signal transduction system, involves the RfrA regulator (Chandler *et al.*, 2003). This protein modulates a second high-affinity Mn transport system which acts under Mn-sufficient conditions, but the mode of action of RfrA remains unknown. RfrA has no sequence or structural similarities to previously described bacterial manganese-regulated transcription factors, and it does not have any known DNA-binding domain. Hence, it is more plausible that RfrA regulates the second  $Mn^{2+}$  transporter through a mechanism other than transcriptional control, such as reversible protein modifications at post-translational level. The regulator contains a conservative repeated-five residues (RFR) domain in the N-terminal, which define a 16-member family in *Synechocystis* PCC 6803. Despite the fact that RFR domains seem to be relatively abundant in other bacterial genomes and especially in photosynthetic organisms, the RFR genes have no defined function (Bateman, Murzin, & Teichmann, 1998). Thus, RfrA becomes the first member of this family of proteins to be linked to a physiological process (Chandler *et al.*, 2003). Further experiments are required to discern the exact mode of RfrA regulation in Mn uptake in cyanobacteria.

### 3.4. The ArsR/SmtB Family of Metal-Sensor Proteins

The SmtB/ArsR proteins function as transcriptional repressors sensing elevated concentrations of different metals not only in cyanobacteria but also in other prokaryotes. This family contains two subfamilies, one comprising SmtB and its orthologues, more divergent than the other subfamily which contains ArsR and its closely related proteins. Similarly, all family members usually control the expression of a metallothionein to sequester metal ions in the cytosol, or an ATPase to export the metal into the periplasm. Conversely, both groups of proteins mainly differ in the metal-binding site (Rensing, 2005).

This protein family includes zinc sensors (SmtB in *Synechococcus* and ZiaR in *Synechocystis*), arsenic, antimony and bismuth (ArsR in *E. coli* and

*Synechocystis*), cadmium, lead and zinc (AztR in *Anabaena* and CadC in *Staphylococcus aureus*), cadmium and lead (CmtR in *M. tuberculosis*), zinc and cobalt (CzrA in *S. aureus*), nickel and cobalt (NmtR and KmtR in *M. tuberculosis*) and copper, silver, zinc and cadmium (BxmR in *Oscillatoria*) (Osman & Cavet, 2010).

One founder member of this family, SmtB, was first cloned and characterized from *Synechococcus* PCC 7942 in 1993. This 122-amino acid protein functions as a Zn(II)-responsive repressor of a metallothionein involved in chelating zinc from cytosol (Huckle et al., 1993). A few years later, an SmtB orthologue was described in *Synechocystis* PCC 6803, named ZiaR. This protein regulates the transcription of an ATPase which exports zinc into the cytoplasm (Thelwell et al., 1998) and it is induced in response to Cd and excess of Zn (Houot et al., 2007). The other founder member, ArsR, was first described as an As(III)/Sb(III)-responsive repressor in *E. coli* (Wu & Rosen, 1991). In *Synechocystis* PCC 6803, an ArsR orthologue is involved in arsenic and antimony resistance, and it thus controls the expression of an operon containing an As(III)/Sb(III)-efflux pump, among other proteins (Lopez-Maury, Florencio, & Reyes, 2003).

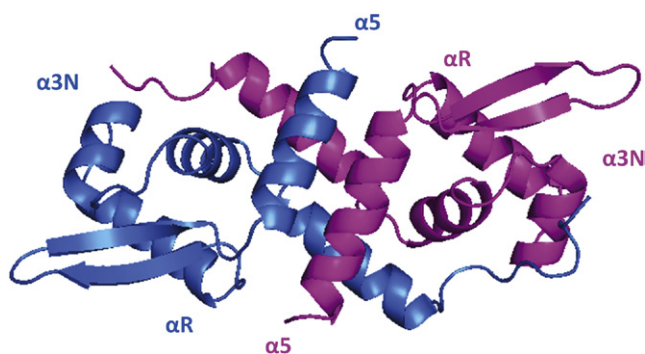
More recently, some other SmtB/ArsR proteins have been discovered in cyanobacteria. *Anabaena* PCC 7120 AztR also represses the transcription of a zinc efflux pump (Liu, Golden, & Giedroc, 2005). A further SmtB/ArsR family member, AzuR, has been reported but not fully characterized (Liu et al., 2008). Notably, in *Oscillatoria brevis*, the Zn(II)-responsive regulator BxmR controls the expression of an ATPase and a metallothionein, but both products are encoded in two physically separate transcription units (Liu et al., 2004). These metal homeostasis systems in *Oscillatoria* remind the zinc response machinery present in mammalian cells with MTF1, which also regulates a metallothionein and a Zn-efflux pump (Jackson, Valentine, Coneyworth, Mathers, & Ford, 2008).

### 3.4.1. Metal-binding sites of SmtB/ArsR family members

The X-ray crystallographic structure of *Synechococcus* apo-SmtB at 2.2 Å resolution shows this regulator as an elongated dimer consisting of two monomers related by a twofold axis of symmetry (Fig. 4.4). Each monomer contains five  $\alpha$ -helices and two  $\beta$ -strands in an  $\alpha\alpha\alpha\beta\beta\alpha$ -fold. Two of the helices,  $\alpha_3$  and  $\alpha_4$  ( $\alpha$ R), form the standard HTH motif present in many DNA-binding proteins. SmtB has strong structural similarities to other HTH transcriptional regulators such as CAP protein or DtxR (Cook et al., 1998).

Analysis of mercuric acetate-soaked crystals suggests the presence of four putative  $\text{Zn}^{2+}$ -binding sites per dimer. It has been proposed that one of them is formed by a molecule of water and residues from each monomer near the  $\alpha 3$  helices: Cys<sup>61</sup>, Asp<sup>64</sup> and His<sup>97</sup>. The other  $\text{Zn}^{2+}$ -binding site might involve residues Asp<sup>104</sup> and His<sup>106</sup> from one monomer and His<sup>117</sup> and Glu<sup>120</sup> from the other monomer, bridging  $\alpha 5$  helices (Cook *et al.*, 1998). The proposed DNA recognition  $\alpha$ -helix ( $\alpha R$ ) is highly conserved among the SmtB/ArsR family members and it confers a high degree of sequence identity (25–50%), allowing the generation of models of SmtB/ArsR repressors based on the *Synechococcus* SmtB crystal structure (Busenlehner, Pennella, & Giedroc, 2003).

Two characteristic metal-binding sites have been described in SmtB/ArsR family members, the  $\alpha 3N$  and the  $\alpha 5$  sites. It has been proposed that the  $\alpha 3N$ -binding site senses larger, thiophilic metals such as Cd(II) or Pb(II) and contains the highly conserved ELCVCDL sequence named the “metal binding box”, whose cysteine pair may be important for metal recognition (Shi, Wu, & Rosen, 1994). This metal-binding site is not regulatory in SmtB (VanZile, Chen, & Giedroc, 2002b). However, substitution of both cysteine residues in *Synechocystis* ZiaR inhibited metal responses in vivo (Thelwell *et al.*, 1998). Consistent with this, the second cysteine residue, Cys<sup>74</sup>, has been suggested to be a critical metal ligand in *Anabaena* AztR (Liu *et al.*, 2005). In *Synechocystis* ArsR, its “metal binding box” EQCVCDL sequence also contains the pair of cysteine residues suggested to interact with arsenite. To our knowledge, no studies about putative metal-binding sites in

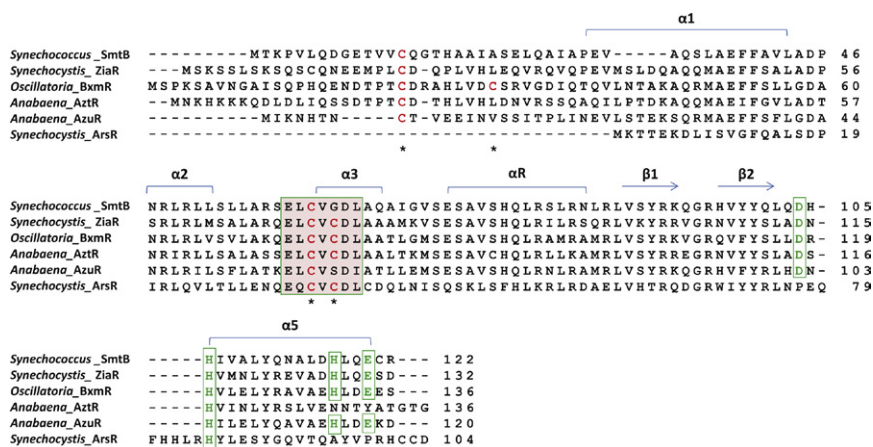


**Figure 4.4** Ribbon diagram of the crystal structure of the apo-SmtB dimer. The structure was imported from UniProt KB (access No 15MT) and drawn with PyMol program (ExPASy server). Each monomer shows an  $\alpha\alpha\alpha\beta\alpha$  fold (Cook *et al.*, 1998). The  $\alpha 5$ ,  $\alpha 3N$  and  $\alpha R$  helices are labelled. Residues from 1 to 24 have been added manually in the figure. For colour version of this figure, the reader is referred to the online version of this book.

cyanobacterial ArsR orthologues have been published. The X-ray structure of apo-SmtB revealed that the  $\alpha 3$  helix contains this metal-binding motif; however, in this regulator, the last cysteine residue is naturally substituted by a glycine residue: ELCVGD $\text{L}$  (Cook et al., 1998). Surprisingly, the remaining cysteine residue in the motif, Cys<sup>61</sup>, is not essential in SmtB for Zn<sup>2+</sup>-sensing in vivo (Turner, Glands, Samson, & Robinson, 1996).

Conversely, the  $\alpha 5$  metal-binding site is composed of ligands derived exclusively from the  $\alpha 5$  helix (Fig. 4.5). Substitution of His<sup>105</sup>/His<sup>106</sup> in SmtB or His<sup>116</sup> in ZiaR by arginine residues in the  $\alpha 5$  helix resulted in a loss of induction by zinc, suggesting that this metal site may be important for Zn<sup>2+</sup>-sensing in vivo for both regulators (Thelwell et al., 1998; Turner et al., 1996). The  $\alpha 5$  metal-binding site probably resists distortion to accommodate larger metal ions, interacting preferably with smaller divalent ions such as Zn(II), Co(II) and Ni(II) (Pennella & Giedroc, 2005).

*Anabaena* AztR lacks the  $\alpha 5$  metal-binding site, and thus, it utilizes the  $\alpha 3\text{N}$  site to sense not only small essential Zn(II) ions but also larger toxic Cd(II)/Pb(II) ions (Liu et al., 2005). The noteworthy structural plasticity of the  $\alpha 3\text{N}$  site has also been shown by its ability to allow direct binding of monovalent ions Cu(I)/Ag(I) in *Oscillatoria* BxmR (Liu et al., 2008). This adaptation of the metal-binding site to different kinds of metals suggests an



**Figure 4.5** Sequence alignment of cyanobacterial SmtB/ArsR family metalloregulators generated using ClustalW2 (<http://www.ebi.ac.uk/Tools/msa/clustalw2/>). The proposed 'metal-binding box' with the Cys-X-Cys motif is boxed and grey shaded. Residues known or predicted to be metal ligands in the  $\alpha 3\text{N}$  site are denoted with an asterisk, and those in the  $\alpha 5$  site are boxed. The secondary structure assignment is based on the studies of SmtB by Cook et al. (1998). For colour version of this figure, the reader is referred to the online version of this book.

evolutionary mechanism developed to confer increased resistance to other toxic heavy metals on certain cyanobacteria.

The sequence of ArsR orthologues does not usually contain the N-terminal region present in SmtB-like proteins. Thus, *E. coli* ArsR possesses three  $\alpha 3$  cysteines in the  $\alpha 3$  helix, which induce metal responsiveness due to the lack of the  $\alpha 5$  site in this regulator. Similarly, *Synechocystis* ArsR does not possess ligands related to the  $\alpha 5$  site either, so it presumably binds metal ions through the  $\alpha 3$  cysteine site (Osman & Cavet, 2010).

An overall comparison of the cyanobacterial SmtB/ArsR repressors reveals distinct metal-binding properties that are summarized in Table 4.5. *Synechococcus* SmtB binds two zinc ions per dimer through the  $\alpha 5$  site (VanZile *et al.*, 2002b). *Synechocystis* ZiaR requires the binding of four zinc ions to both  $\alpha 3N$  and  $\alpha 5$  sites to induce zinc metalloreulation in vivo (Thelwell *et al.*, 1998). *Oscillatoria* BxmR also possesses both sites, using either  $\alpha 5$  or  $\alpha 3N$  to bind zinc ions. However, this regulator affects more selective and effective zinc regulation by binding Zn(II) to the  $\alpha 5$  site, while the  $\alpha 3N$  site is required for Cu(I), Ag(I) or Cd(II) sensing. BxmR binds four Cu(I) ions per dimer in  $\alpha 3N$  site, but only two Zn(II) or Cd(II) ions per dimer in either  $\alpha 5$  or  $\alpha 3N$  sites (Osman & Cavet, 2010). *Anabaena*

**Table 4.5** Summary and comparison of metal-binding properties of cyanobacterial SmtB/ArsR proteins

Sensor	'Metal-binding box'	Metal	Metal site(s)	Ligands
<i>Synechococcus</i> SmtB	ELCVGDL	Zn(II)	$\alpha 5$	Asp104 His106 His117 Glu120
<i>Synechocystis</i> ZiaR	ELCVCDL	Zn(II)	$\alpha 5$ and $\alpha 3N$	$\alpha 5$ : Asp114 His116 His127 Glu130 $\alpha 3N$ : Cys71 and/or Cys73 Cys His
<i>Oscillatoria</i> BxmR	ELCVCDL	Zn(II)  Cu(I), Ag(I), Cd(II)	$\alpha 5$ and $\alpha 3N$  $\alpha 3N$	$\alpha 5$ : Asp119 His121 His132 Glu135 $\alpha 3N$ : Cys23 Cys31 Cys75 Cys77
<i>Anabaena</i> AztR	ELCVCDL	Zn(II) Cd(II) Pb(II)	$\alpha 3N$	$\alpha 3N$ : Cys21 Cys72 Cys74 His
<i>Anabaena</i> AzuR	ELCVSDL	Zn(II)? Co(II)?	$\alpha 5?$	?
<i>Synechocystis</i> ArsR	EQCVCDL	As(III) Sb(III)	$\alpha 3$	?

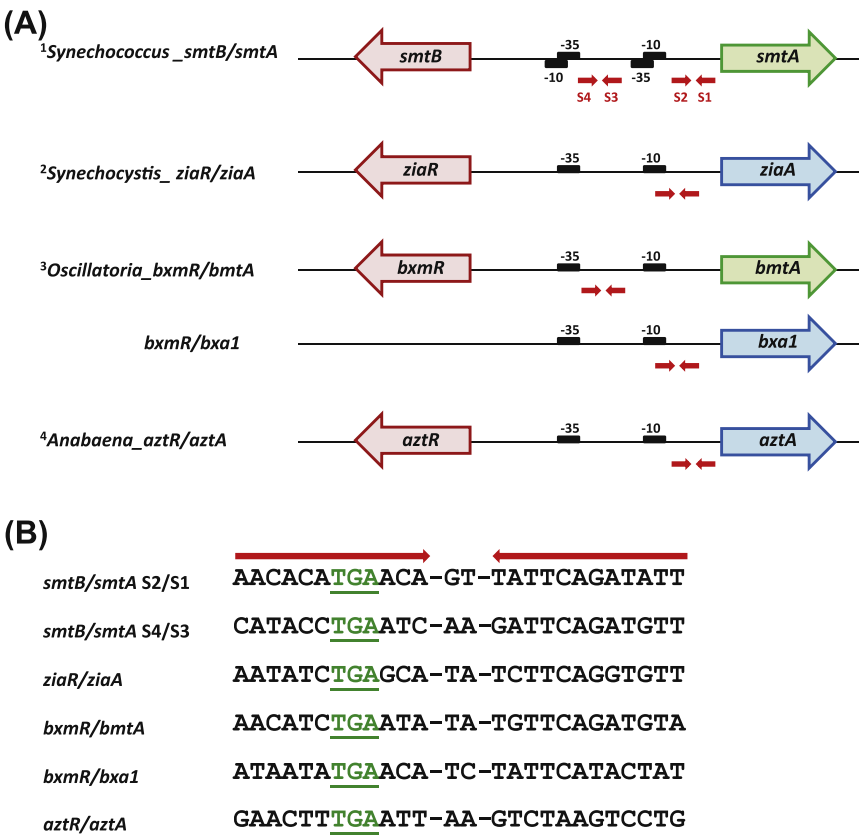
AztR only retains the two  $\alpha$ 3N sites per dimer, so it senses not only two Zn(II) ions but also two Cd(II) and Pb(II) ions per homodimer through this site (Liu et al., 2005). AzuR is the other metalloregulator in *Anabaena* that belongs to the SmtB/ArsR family. This has been proposed to be a zinc repressor more closely related to SmtB (Liu et al., 2008). Finally, *Synechocystis* ArsR is induced in vivo by As(III) and Sb(III), but not by As(V) (Lopez-Maury et al., 2003).

### 3.4.2. DNA-binding sites

The SmtB/ArsR transcriptional repressors often regulate expression of divergently transcribed genes, arranged in operons. These metalloregulators are specifically bound to their DNA operator/promoter (O/P)-binding sites in the metal-free state.

Most of the O/P sequences in this family contain one imperfect 12-2-12 inverted repeat, generally overlapping or located near the transcriptional start site of the genes (Fig. 4.6). For instance, only one pair of contact sites is described for *Synechocystis* ZiaR or *Anabaena* AzrR within the *zia* and *azt* divergons, respectively (Fig. 4.6A) (Liu et al., 2005; Thelwell et al., 1998). Nonetheless, in *Synechococcus*, the *smt* operon displays two of these repeats contacted by SmtB at the conserved TGA sequence: S2/S1 lies at the *smtA* transcriptional start site while S4/S3 lies between the *smtA* and *smtB* –10 sequences. At low concentrations, recombinant SmtB binds in vitro as a monomer to either S1 or S2; as the SmtB concentration increases, it binds as a dimer at both sites. At high SmtB concentrations, the metal-free regulator may form a homotetrameric complex. The formation of all the three DNA-SmtB complexes is inhibited by  $\text{Zn}^{2+}$  in vitro (Erbe, Taylor, & Hall, 1995). In *Oscillatoria*, there is one imperfect 12-2-12 inverted repeat in the region between *bxmR* and *bmtA*, and another one found in the promoter region of *bxal* (Fig. 4.6B) (Liu et al., 2004). The *Synechocystis arsBHC* operon is repressed by the product of a physically separated gene, ArsR. The ArsR-binding site within the *arsBHC* O/P region contains two 17-bp direct repeats of the sequence ATCAAGTTTTTTTGATG, each one consisting of two inverted repeats (Lopez-Maury et al., 2003).

*Synechococcus* mutants with an interrupted *smt* divergon are fivefold sensitive to  $\text{Zn}^{2+}$  and show some reduction in tolerance to  $\text{Cd}^{2+}$  (Turner, Morby, Whitton, Gupta, & Robinson, 1993). The same behaviour is observed in *Synechocystis zia* divergon mutants and both operons seem to be interchangeable. Restoration of metal resistance is achieved by the introduction of *zia* in the *Synechococcus smt* mutant (Thelwell et al., 1998).



**Figure 4.6** A. Organization of the operons encoding cyanobacterial metal-regulated SmtB repressors. Genes encoding metalloregulators (dotted arrows), ATPase genes (grey arrows) and genes encoding metallothioneins (white arrows) are represented. Black arrows indicate the imperfect inverted repeats where the SmtB regulators are bound. (<sup>1</sup>(Erbe *et al.*, 1995); <sup>2</sup>(Thelwell *et al.*, 1998); <sup>3</sup>(Liu *et al.*, 2004); <sup>4</sup>(Liu *et al.*, 2005)). B. Alignment of the DNA-binding sites containing the 12-2-12 inverted repeat sequences from the operons controlled by cyanobacterial SmtB metalloregulators. The conserved sequence TGA which is supposed to be in contact to the regulators is underlined. See the colour plate.

**3.4.3. Functions of SmtB proteins**

In the absence of zinc, *Synechococcus* SmtB is bound to a specific region of the *smt* operon, repressing the divergent transcription of both itself and the gene *smtA* encoding the metallothionein SmtA (VanZile, Chen, & Giedroc, 2002a). When metal availability increases, two zinc ions are bound to the  $\alpha 5$  site in the homodimer triggering transcription until no zinc is left to bind to newly synthesized SmtB and repression is again effective. *Synechococcus smtA* mutants exhibited a fivefold reduction in zinc



tolerance (Turner et al., 1993). SmtA scavenges excess zinc from adventitious sites sequestering it in a nontoxic form. However, overexpressing *smtA* mutants are viable, implying that SmtA does not remove zinc from advantageous sites (Turner, Robinson, & Gupta, 1995). The release of zinc from SmtA has not yet been elucidated. Interaction with another protein or even SmtA degradation has been proposed as zinc release possibilities (Robinson, Whitehall, & Cavet, 2001). In addition to zinc detoxification, another function in zinc accumulation has been suggested for the metallothionein SmtA. The DNA-primase gene, *dnaG*, is located adjacent to *smtA* in *Synechococcus* PCC 7942. It is not known whether SmtA can influence the zinc content of the predicted zinc-finger present in this DNA-primase (Robinson et al., 2001).

The *Synechocystis* *zia* operon is organized in a similar way to the *smt* region; however, ZiaR controls the transcription of a zinc-exporting ATPase ZiaA. In the absence of zinc, the operon is repressed but when metal availability increases, the expression of both the regulator ZiaR and the ATPase ZiaA triggers zinc efflux into the periplasm until levels are low enough to arrest *ziaA* transcription. In addition to zinc hypersensitivity, reduced zinc export to the periplasm is observed in *ziaA* mutants (Barnett et al., 2012; Thelwell et al., 1998).

Thus, two alternative ways of zinc detoxification are represented in these two cyanobacteria: metal sequestration in cytosol by a metallothionein or metal export to the periplasm by an ATPase. In *Oscillatoria*, both systems are present since BxmR metalloregulates not only the expression of the metallothionein BmtA but also the transcription of the ATPase Bxa1. As soon as Zn(II) or Cd(II) are sensed by BxmR, the transporter Bxa1 is firstly induced in a rapid response to restore the intracellular metal homeostasis, while BmtA is transcribed relatively slowly as a long-term defence against metallotoxicity (Liu et al., 2004).

In *Anabaena*, the AztR regulator responds to zinc, cadmium and lead allowing the transcription of the ATPase AztA, which transports divalent ions from cytosol to periplasm (Liu et al., 2005). Surprisingly, the genome of this cyanobacterium also encodes the metallothionein BmtA but an associated SmtB/ArsR regulator has not yet been found (Blindauer, 2008). Notably, a second SmtB orthologue AzuR has been described in *Anabaena*, but it has not yet been well characterized either biochemically or functionally (Liu et al., 2008).

In *Synechocystis*, ArsR senses As(III) and Sb(III) ions and regulates the *arsBHC* operon involved in arsenic and antimony resistance. The *arsC* gene encodes a putative arsenate reductase, related to arsenate detoxification,



probably involved in reduction of As(V) to As(III). ArsB is a putative arsenite and antimonite exporter, while no function has been assigned to ArsH (Lopez-Maury *et al.*, 2003).

#### 3.4.4. Allosteric regulation and autoregulation

Direct interaction of the metal ions in the regulatory sites of the SmtB/ArsR repressors negatively regulates the specific operator/promoter-binding affinity *in vitro*. Negative allosteric regulation of DNA binding is a common characteristic of these family members (Liu *et al.*, 2004).

The binding of two  $\text{Zn}^{2+}$  ions to the  $\alpha 5$  site in the dimer interface of *Synechococcus* SmtB probably induces an overall compaction of the repressor from its conformation in the metal-free state. This conformational change in SmtB could disrupt the geometry necessary for the interaction of the regulator with the binding sites (Kar, Adams, Lebowitz, Taylor, & Hall, 1997). In *Synechocystis* ZiaR, binding of the metal to both  $\alpha 3\text{N}$  and  $\alpha 5$  sites seems to be necessary, implying co-operativity between these sites (Thelwell *et al.*, 1998). *Anabaena* AztR lacks the dimer interface metal-binding residues ( $\alpha 5$  site), thus metal binding to the helix–turn–helix is the proposed simple mechanism to induce DNA dissociation (Liu *et al.*, 2005). *Oscillatoria* BxmR is dissociated from specific DNA *in vitro* upon the addition of both monovalent and divalent metal ions. Cu(I), Ag(I) and Cd(II) inhibit BxmR-*bxa1* O/P DNA binding in an equally effective way, using  $\alpha 3\text{N}$  as metal-binding sites. Concerning Zn(II), this metal is capable of functioning through both metal sites although binding to the  $\alpha 5$  site results in a more effective negative allosteric regulation (Liu *et al.*, 2008).

A mechanism of autoregulation has been described for cyanobacterial SmtB-like proteins. Since the SmtB orthologues are encoded in the divergently transcribed operons that are induced by themselves, their expression is also controlled by metal availability. This autoregulatory mechanism allows returning to the repression condition. However, in *Synechocystis*, the *arsR* gene is not autoregulated since it is expressed constitutively at low level. It is worth noting that in the absence of ArsR, normal growth parameters in *Synechocystis* are observed, in spite of the constitutive expression of ArsB which results toxic for *E. coli* when it is overexpressed (Lopez-Maury *et al.*, 2003).

### 3.5. The MerR Family of Proteins

MerR proteins act as dimeric transcriptional activators that may directly interact with RNA polymerase to achieve a functional fit to DNA. A metal-induced DNA-conformational change distorts the operator structure,

allowing RNA polymerase to initiate transcription from a suboptimal promoter, which has indeed become a potent one. MerR proteins are also capable of autoregulating their own expression (Brown, Stoyanov, Kidd, & Hobman, 2003). The MerR family includes sensors of mercury (MerR in transposable elements Tn21 or Tn501), zinc, cadmium and lead (ZntR in *E. coli*), lead (PbrR in *Ralstonia metallidurans*), copper, gold and silver (CueR in *E. coli*, GolS in *Salmonella*) or cobalt (CoaR in *Synechocystis*) (Osman & Cavet, 2010).

It is noteworthy that MerR proteins also act as weak repressors in the absence of the metal despite being activators. Both repression and activation processes occur while MerR proteins are bound at the same DNA region, between the  $-10$  and  $-35$  promoter elements. This promoter sequence presents an unusual structure as it is about 2 bp longer (19–20 bp) than canonical bacterial promoters. It impairs RNA polymerase from functioning until DNA bending and twisting is possible by metal–regulator interaction. A high degree of sequence similarity in the N-terminal DNA-binding region is another important feature among members of the MerR family, containing a predicted HTH motif followed by a long coiled-coil region. A small C-terminal domain confers metal selectivity (Brown et al., 2003).

In Gram-negative bacteria, MerR regulates expression of the *merTP(C/F)AD(E)* operon in response to mercury. This metal resistance operon encodes the putative transporters MerT, MerC, MerF or MerE, a periplasmic protein MerP, a mercuric reductase MerA and a putative repressor MerD. MerR proteins from these transposons are 144-amino-acid long with a much conserved N-terminal sequence and a variable C-terminal region. MerR family members from Gram-positive bacteria differ from those from Gram-negative ones; they share about 37% amino acid identity. Nonetheless, all MerR proteins contain three conserved cysteine residues, suggested to be involved in  $\text{Hg}^{2+}$  coordination.

In cyanobacteria, an MerR orthologue has been identified by two different research groups as CoaR or CorR. *Synechocystis* PCC 6803 *coaR* (*corR*) is divergently transcribed from *coaT* (*corT*), encoding a putative  $\text{Co}^{2+}$  efflux pump. CoaR is a 370-amino-acid long protein with two different domains. The N-terminal domain aligns with MerR proteins, while the C-terminal region shows sequence similarity to precorrin isomerases, involved in the biosynthetic pathway of cobalamin. This vitamin  $\text{B}_{12}$  contains four corrin rings coordinating a cobalt atom. Notably, a Cys–His–Cys C-terminal motif is involved in cobalt sensing. The *coaR*–*coaT* intergenic region contains a 20-bp spacer with the AAACCTTGCATT–N<sub>6</sub>–AATGTTAAGGTTT

inverted repeat sequence. CoaR binds to this DNA region, and it responds to  $\text{Co}^{2+}$  and to  $\text{Zn}^{2+}$  to a lesser extent. There are two proposed activation models. The first, described by García-Domínguez *et al.*, suggests that  $\text{Co}^{2+}$  binds the corrinoid ring and this complex interacts with CoaR to activate it. Conversely, Rutherford *et al.* propose that the metal and the corrinoid ring bind to different domains in CoaR (García-Domínguez, Lopez-Maury, Florencio, & Reyes, 2000; Rutherford, Cavet, & Robinson, 1999).

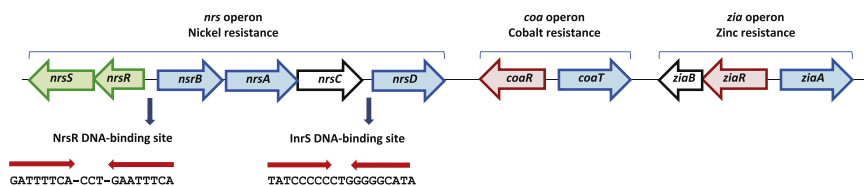
### 3.6. The Nickel-Sensor Proteins

Another important family of metal-sensor proteins is made up of NikR orthologues. In general, NikR proteins act as repressors when bound to DNA in the presence of the co-repressor metal nickel. However, nickel-bound *H. pylori* NikR can also function as a DNA activator. NikR proteins repress the transcription of the *nik* operon, encoding the nickel uptake transporter NikABCDE (Osman & Cavet, 2010). Neither NikR orthologues nor Ni(II)-dependent SmtB/ArsB family members have been identified in cyanobacteria to date.

#### 3.6.1. Nickel-sensing systems in cyanobacteria

Nickel sensing has been best investigated in *Synechocystis* PCC 6803, where two kinds of mechanisms are involved, one controlled by a two-component system that detects periplasmic nickel while the other consists of a cytosolic nickel sensor.

Some years ago, García-Domínguez *et al.* discovered a metal-regulated cluster in *Synechocystis*. This includes the previously described zinc and cobalt response system ZiaR–ZiaA and CoaR–CoaT, respectively (Fig. 4.7).



**Figure 4.7** Genetic organization of the metal-regulated cluster in *Synechocystis*. The different metal-resistance operons and target DNA sequences for nickel resistance are indicated. Genes encoding metalloregulators (dotted arrows), ATPase genes (light grey arrows), genes encoding the two-component system NrsRS (dark grey arrows) and genes with unknown function (white arrows) represented. Direct or inverted repeat sequences present in the NrsR and InrS DNA-binding sites are denoted with arrows (Foster *et al.*, 2012). See the colour plate.

Additionally, the nickel response *nrs* operon, induced in the presence of  $\text{Ni}^{2+}$  and  $\text{Co}^{2+}$ , was also identified. This *nrsBACD* operon encodes a putative membrane-bound protein complex that exports  $\text{Ni}^{2+}$  by a cation/proton antiport (NrsA and NrsB), an unknown function protein (NrsC) and a putative permease belonging to the major facilitator superfamily (MSF), which contains a histidine-rich region involved in  $\text{Ni}^{2+}$  binding (NrsD) (García-Domínguez et al., 2000). Subsequently, *nrsR* and *nrsS* genes were included in this metal-regulated cluster in *Synechocystis*. NrsR and NrsS constitute a two-component signalling system involved in  $\text{Ni}^{2+}$  sensing and in *nrsBACD* operon regulation.

#### 3.6.1.1. Properties of the NrsR/NrsS two-component system

NrsS is a histidine kinase sensor with an N-terminal periplasmic domain that senses periplasmatic nickel. NrsR belongs to the PhoB/OmpR response regulator family with an N-terminal phosphorylation domain and a C-terminal DNA-binding region. It is suggested that the regulator NrsR binds two direct repeats GA(A/T)TTTCA separated by 3 bp in the intergenic region near the  $-10$  box of the two operons, in a similar way to the founder member of its family PhoB. The proposed mechanism of action is that NrsS may sense the presence of nickel in the periplasm and transfer a phosphate to NrsR. Phosphorylated NrsR binds to the *nrsRS–nrsBACD* intergenic region inducing its expression (Lopez-Maury, Garcia-Domínguez, Florencio, & Reyes, 2002).

#### 3.6.1.2. The InrS repressor

Recently, a new cytosolic-nickel sensor, InrS, has been described. This protein represses the expression of the permease NrsD, which exports nickel to the periplasm from a promoter in the *nrsC–nrsD* intergenic region. In *Synechocystis*, InrS possesses a histidine-rich motif in the N-terminal region, and it responds to nickel and cobalt but not to copper. It has been proposed that InrS binds two inversed repeats containing several G/C flanked by an A/T-rich sequence (Foster, Patterson, Pernil, Hess, & Robinson, 2012).

Overall, when  $\text{Ni}^{2+}$  accumulates in the periplasm of *Synechocystis*, the *nrsBACD* operon is expressed under the control of the NrsRS system, allowing nickel efflux across the outer membrane. Meanwhile, cytosolic  $\text{Ni}^{2+}$  is sensed by InrS inducing the expression of the NrsD permease that exports nickel from the cytosol.

### 3.7. Metal Sensors and Nitrogen Metabolism

Nitrogen assimilation and biological nitrogen fixation require a large number of metalloproteins, and changes in the availability of transition metals pose a particular challenge to the supply of these critical nutrients (Glass, Wolfe-Simon, & Anbar, 2009). Nitrogen control in cyanobacteria is mediated by NtcA and the signal transduction P<sub>II</sub> protein, but neither of them are metalloproteins. As mentioned previously, some key genes involved in nitrogen fixation and heterocyst development are co-ordinately modulated by NtcA and FurA, the latter also sensing iron availability.

Molybdenum is a key element in several enzymes involved in nitrogen assimilation and fixation, sulphur, and carbon metabolism. Nitrogenase requires Mo as part of its metal cofactor, and diazotrophic growth of *Anabaena variabilis* has shown to be dependent on the presence of Mo or V, with little growth occurring in their absence (Herrero, Muro-Pastor, & Flores, 2001). In *Azotobacter*, Mo represses the synthesis of both V nitrogenase and nitrogenase-3, and in the absence of Mo, V represses the synthesis of nitrogenase-3 (Luque & Pau, 1991). Molybdenum metabolism and homeostasis are regulated by the molybdate-responsive transcription factor ModE. Orthologues of ModE are widespread amongst diverse prokaryotes but not ubiquitous, and DNA-binding motifs have been identified to be quite conserved (Studholme & Pau, 2003). Little is known about Mo-dependent transcriptional regulators in cyanobacteria. Putative *modE*-like genes have been described in several cyanobacterial genomes (Nakamura *et al.*, 1998), but little information is available in the current literature concerning the role of this regulator in cyanobacteria.



## 4. CONCLUSIONS AND PERSPECTIVES

Metalloregulation in cyanobacteria is not restricted to ensuring optimal metal ion homeostasis. The coordination of metal homeostasis with the response to environmental stresses and central metabolic processes is often carried out by metalloproteins with regulatory functions. Although most of the main families involved in these tasks have been characterized in recent years, further work based on functional genomics and structural biology remains to be done. The identification of new players, such as potential metal-binding noncoding RNAs, will provide a more complete picture of the cyanobacterial metallome.

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# Genomics of the Pleiotropic Glutathione System in Cyanobacteria

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## Abstract

Cyanobacteria, only prokaryotes capable of oxygenic photosynthesis, are fascinating microorganisms that are logically attracting a growing attention in various areas of basic and applied researches. Cyanobacteria have colonized most water and soil environments. Consequently, they support a large part of life on Earth in renewing the oxygenic atmosphere and making up organic assimilates for the food chain. Furthermore, cyanobacteria are regarded as promising 'low-cost' microbial cell factories for carbon

capture and storage, and the sustainable production of biofuels, thanks to their (1) simple nutritional requirements, (2) physiological robustness, (3) metabolic plasticity, and (iv) the powerful genetics of some model strains. Because of their photoautotrophic lifestyle, cyanobacteria are inevitably challenged by toxic reactive oxygen species generated by photosynthesis (and respiration), especially under intense illumination when the light-driven electron transport exceeds what needed for the assimilation of inorganic substrates. This review summarizes what is known regarding the defences against oxidative stress in cyanobacteria, emphasizing on the central role of glutathione and the wealth of glutathione-dependent enzymes, which have been well conserved throughout evolution. We also report on what can be inferred in this field by mining the information provided by the 70 sequenced genomes of morphologically and physiologically diverse cyanobacteria.



## 1. INTRODUCTION

### 1.1. The Fundamental and Biotechnological Interests of Cyanobacteria

Cyanobacteria, formerly termed blue-green algae, are the only prokaryotes that perform the oxygen-evolving photosynthesis. These ancient microorganisms ( $\sim 3 \times 10^9$  years) are regarded as the progenitors of the oxygen-rich atmosphere of our planet (Schopf, 2011) that enabled the evolution of the vital respiration pathway, and as the ancestors of the plant chloroplast (Archibald, 2009). In colonizing most waters (fresh, brackish and marine) and terrestrial (including deserts) environments, cyanobacteria have evolved as the largest and most diverse groups of bacteria (Shi & Falkowski, 2008). They display different forms ranging from unicellular morphologies (spherical and cylindrical) to complex multicellular (filamentous) forms, which can fix atmospheric nitrogen ( $N_2$ ) and establish symbioses with other organisms (fungi, bryophytes, gymnosperms, angiosperm, and the water fern *Azolla filiculoides*). Consequently, cyanobacteria are good model systems to study the impact of changing conditions on the physiology (Battchikova *et al.*, 2010; Hagemann, 2011; Kirilovsky, 2010; Singh *et al.*, 2009), morphology (Vermaas *et al.*, 2008), division (Marbouty, Saguez, Cassier-Chauvat, & Chauvat, 2009; Miyagishima, Wolk, & Osteryoung, 2005) and differentiation (Mariscal & Flores, 2010) of microbial cells. Furthermore, cyanobacteria being the most abundant photosynthetic organisms on Earth (Scanlan *et al.*, 2009), they support a large part of the biosphere in using solar energy to (1) renew the oxygenic atmosphere (Partensky, Hess, & Vaulot, 1999); (2) play important roles in global biogeochemical cycles (assimilation and sequestration of carbon and nitrogen); and (3) make up organic assimilates essential to the food chain. Cyanobacteria convert captured solar energy



into biomass in the field at greater efficiencies (3–9%) than terrestrial plants (0.25–3%), and they tolerate higher CO<sub>2</sub> content in gas streams than plants (Ducat, Way, & Silver, 2011). On a global scale, cyanobacteria fix an estimated 25 Gt of carbon from CO<sub>2</sub> per year into energy-dense biomass. Hence, cyanobacteria are regarded as promising ‘low-cost’ microbial cell factories for the capture and storage of industrial CO<sub>2</sub> gas (Jansson & Northen, 2010), and the ecologically responsible production of biofuels (Ducat et al., 2011; Zhou & Li, 2010). Moreover, cyanobacteria also have the potentials for the biotechnological production of natural products and secondary metabolites (Pearson, Mihali, Moffitt, Kellmann, & Neilan, 2010; Wang, Fewer, & Sivonen, 2011), including vitamin-rich food and therapeutics (Williams, 2009), isotope-labelled molecules and bioplastics (Abed, Dobretsov, & Sudesh, 2009); and for the bioremediation of polluted soil and waters (De Philippis, Colica, & Micheletti, 2011). All these potentials benefit from the capacity of cyanobacteria to grow in a variety of locations. In turn, this enables industrial productions to be performed near the sites of use, thereby reducing transportation costs.

## 1.2. Size, Organization and Ploidy of Cyanobacterial Genomes

The two first articles reporting the sequence of an entire bacterial genome (*Haemophilus influenzae*, 1,830,137 bp; *Mycoplasma genitalium*, 580,070 bp) appeared in 1995 (Fleischmann et al., 1995; Fraser et al., 1995). Very shortly thereafter, the sequence of the 3,573,470-bp chromosome (not of the plasmids at that time) of the widely used unicellular cyanobacterium *Synechocystis* PCC 6803 was published (Kaneko et al., 1996), and modern comparative genomics was born. The number of fully sequenced genomes grew rapidly. Currently, about 50 complete and 20 draft sequences of cyanobacterial genomes are accessible in public data bases such as Cyanobase (<http://genome.kazusa.or.jp/cyanobase/>), DOE joint genome institute (<http://genome.jgi.doe.gov/genome-projects>), and Microbial Genome Database for Comparative Analysis (<http://mbgd.genome.ad.jp/>), and an ever increasing number of cyanobacterial genes are being sequenced in the frame of metagenomic analyses (Hess, 2011; Scanlan et al., 2009; Wang et al., 2011). These data allow to determining which genes are present in any particular genome and which ones are absent. In turn, these information are crucial for genome-based reconstruction of an organism’s metabolism as recently done for the model cyanobacterium *Synechocystis* PCC 6803 (Yoshikawa et al., 2011), and reconstructions of genome evolution



(Price et al., 2012). However, it is important to bear in mind that the bio-informatic method of ‘prediction by analogy’ has its limits. Until we do the experiments, we may mispredict the real function or properties of a gene product (Bender, 2011; Domain, Houot, Chauvat, & Cassier-Chauvat, 2004; Figge, Cassier-Chauvat, Chauvat, & Cerff, 2001).

The genome of cyanobacteria appeared to be widely diverse (Table 5.1), probably as the results of gain and loss of genes transferred by cyanobacterial plasmids, insertion sequences and cyanophages (see below in section 1.2). Most cyanobacteria possess one circular chromosome with a size ranging from about 1.44 Mb in the marine strain UCYN-A (a general trend is that marine species have smaller chromosomes) to about 9.05 Mb in the facultative symbiont *Nostoc punctiforme* ATCC29133 (Hess, 2011; Wang et al., 2011). In addition, many cyanobacteria display a small number of plasmids (a few kilobase to several hundreds of kilobase in size). For instance, *Synechocystis* PCC 6803 possesses seven plasmids, ranging from 2.3 kb (Chauvat, de Vries, Van der Ende, & Van Arkel, 1986) to 119 kb (see CyanoBase; <http://genome.kazusa.or.jp/cyanobase/>). By contrast, the marine cyanobacteria *Prochlorococcus* and *Synechococcus* have no plasmids (Hess, 2011). This finding confirms that marine species have smaller genomes (smaller chromosomes and no plasmids). Very interestingly, the unicellular cyanobacterium *Cyanothece* ATCC51142, which performs photosynthesis during the day and nitrogen fixation at night, harbours a complex 5,460,377-bp genome (Welsh et al., 2008). It comprises a circular chromosome (4,934,271 bp), a smaller linear chromosome (429,701 bp), and four plasmids (ranging from 10,244 to 39,620 bp). The 429,701-bp linear chromosome is the only linear genetic element reported in any photosynthetic bacterium so far. It contains a much higher percentage of genes with no assigned function (71.7% vs. 45.7%) than the large circular chromosome. Nevertheless, it is reasonable to assume that the linear chromosome might be important for carbon metabolism (fermentation among other processes) as it possesses genes presumably involved in glucose and pyruvate metabolisms (Welsh et al., 2008). Similarly, the 8,361,599-bp genome of the chlorophyll d-possessing marine cyanobacterium *Acaryochloris marina* MBIC1101 is also complex. More than 25% of its 8462 genes are distributed over nine plasmids (ranging from 2.13 to 374 kb in size), some of which harbouring presumptive DNA repair genes *recA*, *umuC* and *umuD* (Swingley et al., 2008). Though cyanobacterial plasmids propagate possibly important genes (CyanoBase), their role in the biology and physiology of cyanobacteria remain cryptic, so far.

**Table 5.1** Genomic and metabolic characteristics of cyanobacteria used in this chapter

Species	Isolation site	Order	N <sub>2</sub>	H	M	Features	Size	%GC
<i>Acaryochloris marina</i> MBIC11017	Symbiotic; in coral in Palau republic	C	+	+			8.36	47
<i>Cyanothece</i> sp. ATCC51142	Intertidal areas in Texas	C	+	+			5.46	41
<i>Cyanothece</i> sp. PCC 7424	Rice fields in Senegal	C	?	+			6.82	39
<i>Cyanothece</i> sp. PCC 7425	Rice fields in Senegal	C	?	+			5.82	51
<i>Cyanothece</i> sp. PCC 7822	Rice fields in Cuttack India	C	+	+			7.84	40
<i>Cyanothece</i> sp. PCC 8801	Rice fields in Taiwan	C	+	+			4.81	40
<i>Cyanothece</i> sp. PCC 8802	Rice fields in Taiwan	C	+	+		Need bicarbonate	4.83	40
<i>Microcystis aeruginosa</i> NIES-843	Lake Kasumigaura in Japan	C	+	+		Anaerobic growth	5.84	42
<i>Synechococcus elongatus</i> PCC 6301	Fresh water	C	+	+	–		2.70	55
<i>Synechococcus elongatus</i> PCC 7942	Fresh water	C	–	+	–		2.75	55
<i>Synechococcus</i> sp. CC9311	California current Pacific (95 m)	C	–	–			2.61	52
<i>Synechococcus</i> sp. CC9605	California current Pacific ocean	C	–	–			2.51	59
<i>Synechococcus</i> sp. CC9902	Coastal	C	–	–		Mot	2.23	54
<i>Synechococcus</i> JA-2-3B'a(2-13)	Yellowstone park B-Prime	C	–	–			3.04	58
<i>Synechococcus</i> JA-3-3B' Ab	Yellowstone park B-Prime	C	–	–			2.93	60
<i>Synechococcus</i> sp. PCC 7002	Onshore, Magueyes, Puerto Rico	C	–	+	+		3.40	50

Continued

**Table 5.1** Genomic and metabolic characteristics of cyanobacteria used in this chapter—cont'd

Species	Isolation site	Order	N <sub>2</sub>	H	M	Features	Size	%GC
<i>Synechococcus</i> sp. RCC 307	Mediterranean sea	C	—	—			2.22	60
<i>Synechococcus</i> sp. WH 7803	Sargasso sea Atlantic ocean (25 m)	C	—	—			2.36	60
<i>Synechococcus</i> sp. WH 8102	Caribbean sea	C	—	—		Mot	4.83	59
<i>Synechocystis</i> sp. PCC6803	Freshwater lake California USA	C	—	+	+	Euryhaline, Mot	3.95	48
<i>Thermosynechococcus elongatus</i> BP-1	Beppu hot spring in Japan	C	—	—	—	Thermophilic	2.59	54
<i>Cyanobacterium</i> UCYN-A	North Pacific subtropical	C	+	—		PSII-, CBC	1.44	31
<i>Gleobacter violaceus</i> PCC 7421	Calcareous rock in Switzerland	C	—	—		PS in PM	4.48	62
<i>Anabaena variabilis</i> ATCC 29413	Freshwater sewage oxidation pond	N	+	+	+		7.07	41
<i>Nostoc punctiforme</i> PCC 73102	Symbiosis with cycad Macrorozamia	N	+	—		Ho, Het, Ak	9.01	41
<i>Nostoc</i> sp. PCC 7120	Freshwater USA	N	+	+	+	Het	7.20	41
<i>Nostoc azollae</i> 0708	Water fern <i>Azolla filiculoides</i>	N	+	—		Ho, Het, Ak	5.40	38
<i>Trichodesmium erythraeum</i> ISM101	Coastal waters, North Carolina USA	O	+	—		Ho, Het, Ak	7.80	34
<i>Prochlorococcus marinus</i> AS9601	5 m depth in Mediterranean Sea	P	—	—			1.67	38
<i>Prochlorococcus marinus</i> MIT 9211	North Atlantic ocean (10 m)	P	—	—		LL adapted	1.69	38

**Table 5.1** Genomic and metabolic characteristics of cyanobacteria used in this chapter—cont'd

Species	Isolation site	Order	N <sub>2</sub>	H	M	Features	Size	%GC
<i>Prochlorococcus marinus</i> MIT 9215	Equatorial Pacific HL	P	—	—			1.73	31
<i>Prochlorococcus marinus</i> MIT 9301	Mediterranean sea (5 m)	P	—	—			1.64	31
<i>Prochlorococcus marinus</i> MIT 9303	Mediterranean sea	P	—	—			2.68	50
<i>Prochlorococcus marinus</i> MIT 9312	Gulf Stream surface water	P	—	—		HL adapted	1.71	31
<i>Prochlorococcus marinus</i> MIT 9313	Gulf Stream (135 m)	P	—	—		LL adapted	2.41	51
<i>Prochlorococcus marinus</i> MIT 9515	North Atlantic Ocean (10 m)	P	—	—		LL adapted	1.95	31
<i>Prochlorococcus marinus</i> NATL1A	North Atlantic Ocean (10 m)	P	—	—		LL adapted	1.86	35
<i>Prochlorococcus marinus</i> NATL2A	North Atlantic Ocean (10 m)	P	—	—		LL adapted	1.84	35
<i>Prochlorococcus marinus</i> SS120°	Sargasso sea (120 m)	P	—	—		LL adapted	1.75	36
<i>Prochlorococcus marinus</i> MED4°	Open ocean	P	—	—		HL adapted	1.65	31

Order. C: Chroococcales; N: Nostococcales; O: Oscillatoriales; P: Prochlorales.

N<sub>2</sub>: N<sub>2</sub> fixation.

H<sub>2</sub>: Presence of an NiFe hydrogenase.

M: Able to grow in mixotrophic conditions.

Features. Ak: Akinete; Ho: Hormogony; Het: Heterocyst; Mot: Motility; LL: Low light; HL: High light.

CBC: Calvin Benson cycle; PSII: Photosystem II; PM: Plasma membrane.

S: Genome size.

Chromosomal copy numbers have been investigated in a few cyanobacteria. Marine strains appeared to be monoploid or diploid, in harbouring respectively one or two copies of their chromosome per cell. By contrast, nonmarine strains were found to be polyploid in propagating more than

three copies of their circular chromosome per cell (Griese, Lange, & Soppa, 2011). The figures fluctuate between 3 and 16 for *Synechococcus* PCC 7942 (formerly designated as *Anacystis nidulans*) (Griese et al., 2011; Mann & Carr, 1974) and 12 and 218 for *Synechocystis* PCC 6803 (Griese et al., 2011; Labarre, Chauvat, & Thuriaux, 1989). By contrast, little is known concerning the ploidy of cyanobacterial plasmids, and whether it is possibly influenced by their size, or the nature and growth conditions of their cyanobacterial hosts (Beria & Pakrasi, 2012; Ma, Paulsen, & Palenik, 2012). Recent genomics studies suggest that horizontal gene transfer events might be frequent in cyanobacteria (Hess, 2011). In fresh-water species, two major modes of gene transfer have been identified: natural transformation (Chauvat, Astier, Vedel, & Joset-Espardellier, 1983; Grigorieva & Shestakov, 1982), and conjugation with either cyanobacterial (Wolk, Vonshak, Kehoe, & Elhai, 1984) or non-cyanobacterial plasmids (Mermet-Bouvier, Cassier-Chauvat, Marraccini, & Chauvat, 1993). In addition, insertion sequences and cyanophages could contribute to gene transfers among cyanobacteria. Indeed, several cyanobacterial insertion sequences are truly mobile (Cassier-Chauvat, Poncelet, & Chauvat, 1997), and some cyanophages possess genes presumably involved in photosynthesis (Clokic & Mann, 2006) or anti-oxidant defences (glutaredoxins, see below section 5) that could be involved in or resulting from gene transfer.

### 1.3. Redox Stress in Cyanobacteria

Because of their lifestyle, cyanobacteria are continuously challenged with toxic reactive oxygen species (ROS) present in our oxygenic atmosphere (ozone,  $O_3$ ), or generated by photosynthesis (Kirilovsky, 2010), respiration and cell metabolism (Latifi, Ruiz, & Zhang, 2009). These oxidative agents are, namely, singlet oxygen ( $^1O_2$ ), the superoxide anion ( $O_2^{\cdot-}$ ), hydrogen peroxide ( $H_2O_2$ ), and the hydroxyl radical ( $OH\cdot$ ) (Imlay, 2008). Among other ROS-generated damages, cysteines can be oxidized to form thiyl (sulfenyl) radical ( $-S^\bullet$ ) by one-electron transition; sulfenic acid ( $-SOH$ ) and disulfide ( $-S-S-$ ) by a two-electrons transition; sulfinic acid ( $-SO_2H$ ) by a four-electrons transition; and eventually sulfonic acid ( $-SO_3H$ ) by a six-electrons transition (Forman, Maiorino, & Ursini, 2010). Two types of disulfide can be distinguished considering whether they link two cysteinyl residues, from the same or different proteins (intra- or intermolecular disulfide bridges) or from a protein and a molecule of the anti-oxidant tripeptide glutathione (glutathione-protein mix disulfide, also termed glutathionylation). These sulphur switches can provide an important and flexible means of reversibly controlling protein function. Glutathionylation is regarded as a transient

protection of critical cysteines against irreversible oxidation (sulfinic and sulfonic acids) during oxidative stress (Masip, Veeravalli, & Georgiou, 2006), and as a post-translational regulatory modification (Dalle-Donne, Rossi, Colombo, Giustarini, & Milzani, 2009; Rouhier, Lemaire, & Jacquot, 2008; Zaffagnini, Bedhomme, Marchand, Morisse, Trost, & Lemaire, 2012).

The ROS oxidants can be detoxified by various metabolites (ascorbate, carotenoids, glutathione, vitamins, etc.) and several enzymes. The enzymes – superoxide dismutase (SOD), catalase and peroxidase – sequentially convert the superoxide anion to hydrogen peroxide (SOD) and subsequently hydrogen peroxide to water (catalase and peroxidase) (Imlay, 2008; Latifi et al., 2009; Masip et al., 2006). By contrast, the protein disulfides and glutathione–protein mix disulfides are repaired by thioredoxins (Berndt, Lillig, & Holmgren, 2008) and glutaredoxins (Lillig, Berndt, & Holmgren, 2008; Zaffagnini, Bedhomme, Marchand et al., 2012). If and when the oxidants outnumber the anti-oxidants, the resulting oxidative stress can lead to cell death in microorganisms and pathologies in mammals. In addition, the ROS species, more particularly  $\text{H}_2\text{O}_2$ , can also operate in signalling, which is an important physiological process (Forman et al., 2010). Indeed,  $\text{H}_2\text{O}_2$  possesses the required properties to be a secondary messenger in being enzymatically produced and degraded by the superoxide dismutase and catalase enzymes, respectively. Furthermore,  $\text{H}_2\text{O}_2$  oxidizes protein thiols in disulfides, which can be reduced back to thiols, are thereby relevant as thiol redox switches for signalling.

This review outlines the variety of the processes used by cyanobacteria to protect themselves against oxidative stress, emphasizing on glutathione and the wealth of glutathione-dependent enzymes, which have been well conserved during evolution (Couturier, Jacquot, & Rouhier, 2009; Dalle-Donne et al., 2009; Masip et al., 2006).



## **2. GLUTATHIONE AN INTEGRATIVE LINK BETWEEN CELL METABOLISM AND THE DEFENCES AGAINST OXIDATIVE AND METAL STRESSES**

### **2.1. The Pleiotropic Roles of Glutathione**

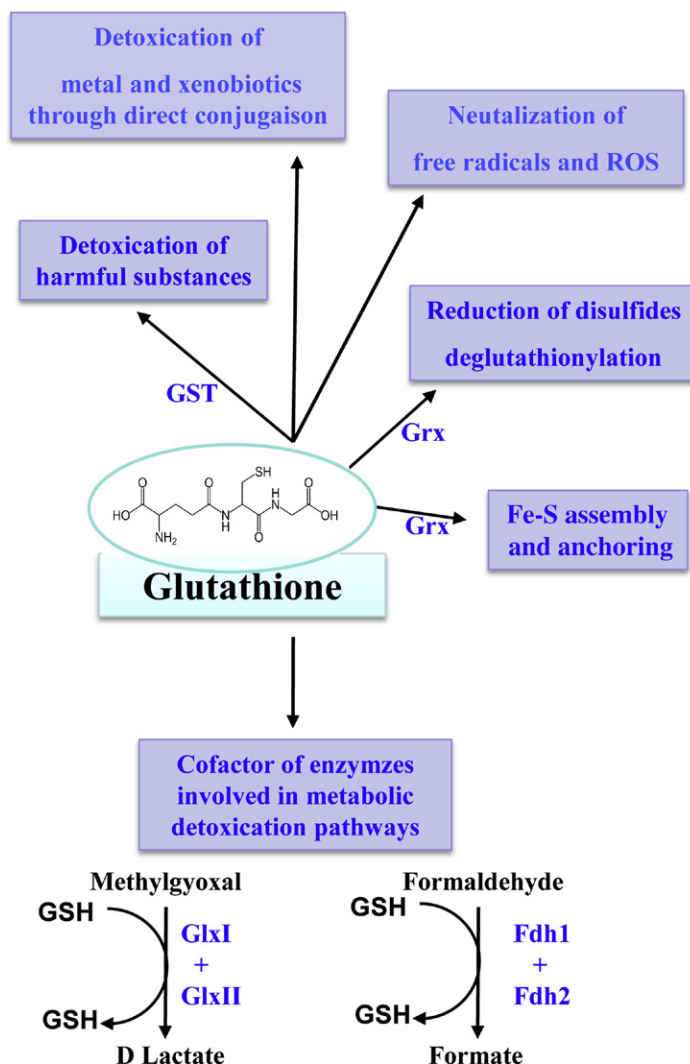
Various metabolites as alpha tocopherol (vitamin E), carotenoids (beta-carotene, myxoxanthophyll, zeaxanthin), and glutathione (the tripeptide  $\gamma$ -L-glutamyl-L-cysteinyl-L-glycine) operate in the defence against ROS (Latifi et al., 2009; Masip et al., 2006). Glutathione (GSH) is one of the most abundant cellular thiols (concentration ranging from 0.1 to about

10 mM) present in cyanobacteria, proteobacteria, a few Gram-positive bacteria, as well as in all mitochondria or chloroplast-bearing eukaryotes (Masip et al., 2006; Zhang & Forman, 2012). As summarized in Fig. 5.1, GSH plays a central role in redox control of protein thiols and disulfide bonds, as well as in protection against toxic metabolites (methylglyoxal and formaldehyde), electrophiles, xenobiotics (Cameron & Pakrasi, 2010; Masip et al., 2006), antibiotics (Cameron & Pakrasi, 2011), and oxidative and osmotic stresses (Masip et al., 2006). In addition, GSH operates in the protection against arsenite (As(III)), a frequent pollutant. In response to As, the yeast *Saccharomyces cerevisiae* exports and accumulates GSH outside the cells where it conjugates with As forming the arsenite triglutathione complex As(GS)<sub>3</sub> that cannot enter cells, which are thereby protected from As toxicity (Thorsen et al., 2012). GSH is also a key component of the cytoplasmic pool of labile iron, mostly occurring under the Fe(II)GSH complex (Hider & Kong, 2011), which likely supplies Fe for the synthesis of the Fe or (Fe–S) cluster cofactors of a wealth of enzymes involved in electron transfers (photosynthesis respiration) and central metabolism. This finding sheds light on the cross-talk between GSH and iron homeostasis, which are especially important in cyanobacteria. Because they possess abundant Fe-requiring machineries for photosynthesis, respiration and nitrogen assimilation of cyanobacteria need an order of magnitude more Fe atoms within their cells than heterotrophic bacteria (Shcolnick, Summerfield, Reytman, Sherman, & Keren, 2009). Furthermore, Fe homeostasis and GSH play a crucial role in the cyanobacterial defence against oxidative and metal stresses (Cameron & Pakrasi, 2010; Houot et al., 2007; Shcolnick et al., 2009).

## 2.2. Biosynthesis of Glutathione

GSH is synthesized by the sequential action of two ATP-requiring enzymes (Fig. 5.2), the  $\gamma$ -glutamyl-cysteine synthetase (GshA) enzyme, which catalyses the addition of glutamic acid to cysteine to form the  $\gamma$ -glutamyl-cysteine product, and the glutathione synthetase (GshB) enzyme, which adds glycine to  $\gamma$ -glutamyl-cysteine to form GSH (Masip et al., 2006). In *Escherichia coli*, GshA, the rate-limiting enzyme for GSH synthesis, is a monomer of 58.3 kDa, while GshB is a tetramer with four identical subunits of 35.6 kDa (Masip et al., 2006). GSH is dispensable in *E. coli* growing under laboratory conditions (Veeravalli, Boyd, Iverson, Beckwith, & Georgiou, 2011), whereas it is essential for cell growth in eukaryotes (Spector, Labarre, & Toledano, 2001).

The comparison of the highly divergent GshA sequences and the less divergent GshB sequences suggests that the evolutionary history of their



**Figure 5.1 The crucial roles of glutathione.** GSH: Glutathione, Grx: Glutaredoxin, GST: Glutathione-S transferase, GlxI: Glyoxalase I, GlxII: Glyoxalase II, Fdh1: Glutathione-dependent formaldehyde dehydrogenase, Fdh2: Formylglutathione hydrolase (esterase).

genes is complex in that *gshA*, which likely arose in cyanobacteria, and *gshB* were acquired independently in present days organisms (Ashida, Sawa, & Shibata, 2005; Copley & Dhillon, 2002). All sequenced cyanobacterial genomes possess the two genes *gshA* and *gshB*, which are never grouped into the same gene cluster in spite of the close relation between the GshA and GshB enzymes (Table 5.2). The cyanobacterial *gshB* gene is mostly





phytochelatin synthase (Alr0975 in the model cyanobacterium *Nostoc* PCC7120 (Tsuji et al., 2004)) to form phytochelatins (( $\gamma$ -Glu-Cys)<sub>2-11</sub>-Gly), which chelates metals (Pandey, Rai, & Rai, 2012) and protects cells from the deleterious effect of UV-B (Bhargava, Srivastava, Urmil, & Rai, 2005).

### 2.3. Degradation of Glutathione

Because of its  $\gamma$ -linkage between the amine group of cysteine and the carboxyl group of glutamate, GSH cannot be degraded by protease. However, one peptidase,  $\gamma$ -glutamyl transpeptidase (GGT), widely distributed among bacteria and eukaryotes, catabolizes GSH (Masip et al., 2006). GGT releases cysteinyl glycine and transfers the glutamyl moiety of GSH to a variety of acceptor molecules as water, thereby regenerating glutamate, and some amino acids and dipeptides (Fig. 5.2). The mammalian GGT enzyme preferentially transfers the glutamyl residue from GSH to cysteine, yielding the  $\gamma$ -glutamyl-cysteine that is rapidly reduced to  $\gamma$ -glutamyl-cysteine and cysteine (Zhang & Forman, 2012). Mammalian GGTs are embedded in the plasma membrane by an N-terminal transmembrane peptide and are heterologously glycosylated. By contrast, bacterial GGTs are generally soluble and localized in the periplasmic space by an N-terminal signal peptide or secreted in the extracellular environment. In *E. coli* and a few other bacteria, another enzyme, the tripeptidase (PepT), operates in GSH degradation, and both the GGT- and the PepT-encoding genes are dispensable for cell growth under favourable laboratory conditions (Lin, Liao, Zhang, Du, & Chen, 2009). We found no *pepT* orthologous gene in cyanobacteria (Table 5.2). Most marine cyanobacteria also lack a GGT-encoding gene, whereas nonmarine strains possess 1–4 presumptive *ggt* genes (Table 5.2). In *Synechocystis* PCC 6803, the *ggt* gene *slr1269* is localized between *slr1267*, which codes for FtsW an important protein involved in cell division (Marbouty, Mazouni, Saguez, Cassier-Chauvat, & Chauvat, 2009), but this genome organization is not shared by other cyanobacteria.

Considering the importance of GSH, and of its polymer phytochelatin, for cyanobacteria, it is urgent to investigate not only its synthesis but also its turnover, and see whether this function involves the presumptive GGT enzymes and/or other as yet unidentified enzymes.

### 2.4. Reduction of Glutathione

Upon the detoxification of ROS, GSH is oxidized as glutathione disulfide (GSSG), which can subsequently be reduced by the NADPH-dependent glutathione reductase enzyme present in most but not all organisms

**Table 5.2** Distribution and genomic organization of genes involved in the metabolism of glutathione in cyanobacteria

Species	Genes and genetic organization					Gst					Grx (CPXC)	S1	Grx3 (CGFS)	S2
	GshA	GshB	Ggt	GR	Σ	a	b	c	d	PCs				
<i>Acaryochloris marina</i> MBIC11017	+	+	1	+	16	+	+	+	+	—	2	+	+	—
<i>Cyanothece</i> sp. ATCC51142	+	+	2	+	5	+	+	+	+	+	2	+	+	A
<i>Cyanothece</i> sp. PCC 7424	+	+	4	+	7	—	+	—	+	—	2	+	+	—
<i>Cyanothece</i> sp. PCC 7425	+	+	—	+	8	+	+	+	+	+	1	+	+	A
<i>Cyanothece</i> sp. PCC 7822	+	+	2	+	2	+	+	—	—	+	1	+	+	—
<i>Cyanothece</i> sp. PCC 8801	+	+	1	+	6	2	—	+	+	—	2	+	+	A
<i>Cyanothece</i> sp. PCC 8802	+	+	1	+	2	2	—	+	—	—	2	+	+	A
<i>Microcystis aeruginosa</i> NIES-843	+	+	1	+	2	2	—	—	—	—	—	2	+	+
<i>Synechococcus elongatus</i> PCC 6301	+	+	1	+	4	+	+	+	+	—	1	+	+	A
<i>Synechococcus elongatus</i> PCC 7942	+	+	2	+	3	+	+	+	—	—	1	+	+	A
<i>Synechococcus</i> sp. CC9311	+	+	—	+	5	+	—	2	+	—	1	+	+	C
<i>Synechococcus</i> sp. CC9605	+	+	1	+	4	+	—	+	+	—	1	+	+	C
<i>Synechococcus</i> sp. CC9202	+	+	1	+	4	+	—	+	+	—	1	+	+	C
<i>Synechococcus</i> JA-2-3B' a(2-13)	+	+	1	—	1	—	—	—	+	—	2	—	+	—
<i>Synechococcus</i> JA-3-3B' Ab	+	+	1	—	1	—	—	—	+	—	2	—	+	—
<i>Synechococcus</i> sp. PCC 7002	+	+	1	—	2	+	—	+	—	—	2	+	+	A
<i>Synechococcus</i> sp. RCC 307	+	+	1	+	5	+	—	+	+	—	1	+	+	C
<i>Synechococcus</i> sp. WH 7803	+	+	1	+	6	+	—	—	+	—	1	+	+	C
<i>Synechococcus</i> sp. WH 8102	+	+	1	+	2	+	—	+	—	—	1	+	+	C
<i>Synechocystis</i> sp. PCC 6803	+	+	1	—	4	2	+	+	—	—	2	+	+	A
<i>Thermosynechococcus elongatus</i> BP-1	+	+	—	+	1	—	—	+	—	—	1	+	+	A
<i>Cyanobacterium</i> UCYN-A	+	+	—	+	+	—	—	+	—	—	1	+	+	B
<i>Gleobacter violaceus</i> PCC 7421	+	+	3	+	5	—	+	—	+	—	1	+	+	—
<i>Anabaena variabilis</i> ATCC 29413	+	+	1	+	10	+	—	—	+	+	2	+	+	A

<i>Nostoc punctiforme</i> PCC 73102	+	+	1	+	15	+	-	+	+	+	2	+	+	B
<i>Nostoc</i> sp. PCC 7120	+	+	1	+	7	+	-	+	5	+	2	+	+	A
<i>Nostoc azollae</i> 0708	+	+	-	+	2	2	-	-	-	-	2	+	+	B
<i>Trichodesmium erythraeum</i> ISM101	+	+	2	+	5	+	-	-	+	+	2	+	+	B
<i>Prochlorococcus marinus</i> AS9601	+	+	-	+	4	+	-	-	+	-	1	+	+	C
<i>Prochlorococcus marinus</i> MIT 9211	+	+	1	+	4	+	-	-	+	-	1	+	+	C
<i>Prochlorococcus marinus</i> MIT 9215	+	+	-	+	4	+	-	-	+	-	1	+	+	C
<i>Prochlorococcus marinus</i> MIT 9301	+	+	-	+	4	+	-	-	+	-	1	+	+	C
<i>Prochlorococcus marinus</i> MIT 9303	+	+	-	+	4	+	+	-	+	+	1	+	+	C
<i>Prochlorococcus marinus</i> MIT 9312	+	+	-	+	3	+	-	-	+	-	1	+	+	C
<i>Prochlorococcus marinus</i> MIT 9313	+	+	-	+	2	+	+	-	-	+	1	+	+	C
<i>Prochlorococcus marinus</i> MIT 9515	+	+	-	+	+	+	-	-	-	-	1	+	+	C
<i>Prochlorococcus marinus</i> NATL1A	+	+	-	+	5	+	-	-	+	-	1	+	+	B
<i>Prochlorococcus marinus</i> NATL2A	+	+	-	+	2	+	-	-	+	-	1	+	+	B
<i>Prochlorococcus marinus</i> SS120	+	+	1	+	3	+	-	-	-	-	1	+	+	C
<i>Prochlorococcus marinus</i> MED4	+	+	1	+	3	+	-	-	+	-	1	+	+	C

GshA:  $\gamma$ -glutamate-cysteine ligase.

GshB: glutathione synthase.

Ggt:  $\gamma$ -glutamyltranspeptidase.

Gor: glutathione reductase.

Gst: glutathione S-transferase;  $\Sigma$ : number of Gst a = Gst homologous to *Synechocystis* Slr0236 et Sll1545.

b = membrane-bound Gst homologous to *Synechocystis* Sll1147.

c = Gst homologous to *Synechocystis* Sll0067.

d = other type of GST.

PC: phytochelatin synthase.

Grx (CPXC): dithiol glutaredoxin.

Grx (CGFS): monothiol glutaredoxin (grx3).

S1: genomic organization; syntheny *grx-gshB*.

S2: genomic organization cluster *bolA-grx3*.

A = syntheny *bolA-grx3*.

B = syntheny *ho-bolA-grx*.

C = syntheny *plsC-hobolA-grx*.

plsC: phospholipid/glycerol acyltransferase.

(Marteyn, Domain, Legrain, Chauvat, & Cassier-Chauvat, 2009). The GSH/GSSG molecular ratio is about 200 in *E. coli* cells growing in the rich standard-medium LB (Masip et al., 2006), and even higher in plants (Zaffagnini, Bedhomme, Marchand et al., 2012). Because the NAD(P)H/NAD(P) and GSH/GSSG systems do not exchange electrons directly at any appreciable rate, the two redox couples can be maintained within the cell at different redox potentials as required for cellular processes (Masip et al., 2006).

Four unicellular cyanobacteria apparently lack a glutathione reductase encoding gene, namely the two euryhaline strains *Synechococcus* PCC 7020 and *Synechocystis* PCC 6803, and the two *Synechococcus* strains isolated in Yellowstone (Table 5.2). In the filamentous cyanobacterium *Anabaena* PCC 7120, the glutathione reductase gene (*all4968* in CyanoBase) is expressed from two *E. coli*-like sigma70-type promoters, which are active alternatively or in combination, depending on the nitrogen source (Jiang, Hellman, Sroga, Bergman, & Mannervik, 1995). The *all4968* gene, which has been cloned and expressed in a glutathione reductase-deficient *E. coli* strain, produce an enzyme carrying the GXGXXG fingerprint motif (amino acids 173–178) normally present in NADH-dependent enzymes, instead of the GXGXXA motif occurring in NAD(P)H-dependent enzymes (Danielson, Jiang, Hansson, & Mannervik, 1999; Jiang et al., 1995). It will be very interesting to study and compare the pleiotropic roles of GSH in the responses to oxidative and metal stresses in the cyanobacteria *Synechococcus* PCC 7942 and *Synechocystis* PCC 6803, which possess or lack the glutathione reductase enzyme, respectively (Marteyn et al., 2009).



### **3. GLUTATHIONE OPERATES IN THE DEFENCE AGAINST TOXIC ENDOGENOUS METABOLITES**

#### **3.1. Glutathione Operates in the Detoxification of Methylglyoxal**

Methylglyoxal (MG), also called pyruvaldehyde or 2-oxopropanal ( $\text{CH}_3\text{--CO--CH=O}$  or  $\text{C}_3\text{H}_4\text{O}_2$ ), is the aldehyde form of the pyruvic acid. Since MG possesses two carbonyl groups, it is more active than glucose in protein glycation (the nonenzymatic glycosylation), which is the result of, typically covalent, bonding of a sugar molecule with a protein (the free amino groups of lysine and arginine and the thiol group of cysteine). The formation and accumulation of the so-called advanced glycation endproducts (AGEs), which impair protein functions, has been implicated in ageing and the progression of age-related diseases such as diabetes, Parkinson's (Lee et al., 2012) and Alzheimer's (Xue, Rabbani, & Thornalley, 2011) diseases.

In yeast, MG is produced by the spontaneous breakdown of the glyceraldehyde-3-phosphate metabolite (Inoue, Maeta, & Nomura, 2011). By contrast, in bacteria, MG is mainly synthesized enzymatically from the glycolytic intermediate dihydroxyacetone phosphate (DAHP), via the action of MG synthase, which is allosterically controlled by DHAP and feedback inhibited by inorganic phosphate (Masip et al., 2006). In cyanobacteria, only the nonmarine strains possess a presumptive MG synthase-encoding gene (Table 5.3), which appeared to be crucial to cell growth in *Synechocystis* PCC 6803 (Narainsamy, Chauvat and Cassier-Chauvat unpublished results).

GSH plays a major role in cell protection against MG (Fig. 5.1), a function catalysed by the two enzymes glyoxalase I (GlxI) and glyoxalase II (GlxII), which are ubiquitous (Inoue et al., 2011; Lee et al., 2012; Masip et al., 2006; Yadav, Singla-Pareek, & Sopory, 2008). GlxI (S-D-lactoylglutathione methylglyoxal lyase) converts the spontaneously formed hemithioacetal adduct between GSH and MG, to S-D-lactoylglutathione. This glutathione thiolester is then hydrolysed by GlxII (S-2-hydroxyacylglutathione hydrolase) to produce the nontoxic D-lactate and regenerate GSH (Suttisansanee & Honek, 2011). Both GlxI and GlxII are single enzymes in *E. coli* (Masip et al., 2006), whereas the yeast *S. cerevisiae* possesses a single GlxI enzyme and two GlxII enzymes, which are all dispensable for normal growth (Inoue et al., 2011). In bacteria, the metalloenzymes GlxI with shorter amino acid sequences (~130 amino acids in length) tend to be  $\text{Ni}^{2+}/\text{Co}^{2+}$ -activated (Mullings, Sukdeo, Suttisansanee, Ran, & Honek, 2012), while longer GlxI (~180 amino acids in length) are likely  $\text{Zn}^{2+}$ -activated enzymes (Suttisansanee et al., 2011). In yeast, GlxI contains both Fe and Zn (Inoue et al., 2011), and the expression of its gene is regulated by osmotic stress conditions to combat the increased production of MG accompanying the increased synthesis of the glycerol osmolyte (Inoue et al., 2011). GlxII is a metallo-enzyme with binuclear active sites per monomer that can be activated by various metals, depending on the particular source organism. As isolated, *E. coli* GlxII binds 1.7 mol of Zn per mole of monomeric enzyme, while other metals were not detected (Suttisansanee & Honek, 2011).

Besides the GlxI/GlxII system, various organisms have other routes to detoxify MG. In *E. coli*, the heat shock protein Hsp31, renamed as glyoxalase III, directly converts MG to D-lactate without the need for the GSH cosubstrate, unlike the GlxI/GlxII system (Subedi, Choi, Kim, Min, & Park, 2011; Suttisansanee & Honek, 2011). In addition, MG can be reduced to L-lactaldehyde by the NADPH-dependent MG reductase, also present in *S. cerevisiae* (Inoue et al., 2011). Furthermore, MG can be converted to acetol

by its four aldo-keto reductase (AKR) enzymes named YafB, YqhE, YeaE, and YghZ, which are dispensable for *E. coli* growth of Ko et al. (2005). These AKR enzymes belong to a large superfamily of NADPH-dependent oxidoreductases that also occur in eukaryotes, including humans (Mindnich & Penning, 2009) and plants (Turoczy et al., 2011).

All cyanobacteria, except *Synechococcus* JA-2-3B'a(2-13), have at least one presumptive pathway for MG detoxification, mostly the glyoxalase enzymes (Table 5.3), which are dispensable for the normal growth of *Synechocystis* PCC 6803 (Narainsamy, Chauvat and Cassier-Chauvat unpublished results). In addition, many strains also have AKR enzymes (Table 5.3). In *Synechococcus* PCC 7002, a rare strain capable of growing heterotrophically at the expense of glycerol, one of the four presumptive AKR enzymes (A1474) was found to be a genuine, but dispensable, AKR enzyme. The A1474-deleted mutant is sensitive to glycerol, which triggers the accumulation of MG (Xu, Liu, Guo, & Zhao, 2006).

### 3.2. Glutathione Operates in the Detoxification of Formaldehyde

Formaldehyde is extremely reactive in producing covalent cross-linked complexes with proteins and nucleic acids. It is not only a common environmental pollutant but also an endogenous compound present in all living organisms as the result of the catabolism of methionine, methanol and glyoxylate; or the oxidative demethylation of DNA and RNA (Gonzalez et al., 2006) and references therein. The GSH-dependent defence system found in most prokaryotes and all eukaryotes (Fig. 5.1) proceeds as follows. Formaldehyde spontaneously reacts with GSH to produce S-hydroxymethylglutathione, which is oxidized by formaldehyde dehydrogenase (Fdh1) to S-formylglutathione, which is hydrolysed by S-formylglutathione hydrolase (Fdh2) to generate formate and GSH. The Fdh2 enzymes from yeasts and *Arabidopsis thaliana* are not strictly specific to S-formylglutathione, in showing significant carboxylesterase activity against the model substrates  $\alpha$ -naphthyl acetate and p-nitrophenyl acetate. It has also been proposed that Fdh2 are cysteinyl hydrolases because the *A. thaliana* enzyme is highly sensitive to the inhibition by N-ethylmaleimide.

The two functionally related Fdh1 and Fdh2 enzymes also show a genetic linkage since their genes are adjacent in the genomes of many bacteria and some cyanobacteria (Table 5.3) or even fused in some eukaryotes. Also interestingly, these genes are absent in the marine Prochlorococcales cyanobacteria (Table 5.3).

**Table 5.3** Distribution and genomic organization of genes involved in the GSH-dependent detoxication of toxic metabolites

Species	MGS	GlxI	GlxII	Akr	Fdh1	Fdh2	S3
<i>Acaryochloris marina</i> MBIC11017	2	+	+	1	+	+	—
<i>Cyanothece</i> sp. ATCC51142	+	+	+	3	+	—	—
<i>Cyanothece</i> sp. PCC 7424	2	+	+	3	+	—	—
<i>Cyanothece</i> sp. PCC 7425	+	+	+	3	+	+	—
<i>Cyanothece</i> sp. PCC 7822	2	+	+	3	+	—	—
<i>Cyanothece</i> sp. PCC 8801	—	+	+	1	+	+	—
<i>Cyanothece</i> sp. PCC 8802	—	+	+	2	+	+	—
<i>Microcystis aeruginosa</i> NIES-843	—	+	+	2	+	—	—
<i>Synechococcus elongatus</i> PCC 6301	—	+	+	1	+	+	+
<i>Synechococcus elongatus</i> PCC 7942	—	+	+	1	+	+	+
<i>Synechococcus</i> sp. CC9311	—	+	+	2	+	+	+
<i>Synechococcus</i> sp. CC9605	—	+	+	1	—	—	—
<i>Synechococcus</i> sp. CC9202	—	+	+	1	—	—	—
<i>Synechococcus</i> JA-2-3B' a(2-13)	—	—	—	—	—	—	—
<i>Synechococcus</i> JA-3-3B' Ab	—	+	+	—	—	—	—
<i>Synechococcus</i> sp. PCC 7002	+	+	+	1	+	+	+
<i>Synechococcus</i> sp. RCC 307	—	+	+	—	—	—	—
<i>Synechococcus</i> sp. WH 7803	—	+	+	—	+	+	+
<i>Synechococcus</i> sp. WH 8102	—	+	+	—	—	—	—
<i>Synechocystis</i> sp. PCC 6803	+	+	+	3	+	+	+
<i>Thermosynechococcus elongatus</i> BP-1	+	—	+	—	—	—	—
<i>Cyanobacterium</i> UCYN-A	—	+	+	—	—	—	—
<i>Gleobacter violaceus</i> PCC 7421	2	+	+	—	+	+	+
<i>Anabaena variabilis</i> ATCC 29413	+	+	+	3	+	+	+★
<i>Nostoc punctiforme</i> PCC 73102	+	+	+	3	+	+	+
<i>Nostoc</i> sp. PCC 7120	+	+	+	3	+	+	+★

Continued



**Table 5.3** Distribution and genomic organization of genes involved in the GSH-dependent detoxication of toxic metabolites—cont'd

Species	MGS	GlxI	GlxII	Akr	Fdh1	Fdh2	S3
<i>Nostoc azollae</i> 0708	—	+	+	1	+	+	+
<i>Trichodesmium erythraeum</i> ISM101	—	+	+	1	+	+	+★
<i>Prochlorococcus marinus</i> AS9601	—	+	+	1	+	+	+★
<i>Prochlorococcus marinus</i> MIT 9211	—	+	+	—	—	—	—
<i>Prochlorococcus marinus</i> MIT 9215	—	+	+	1	—	—	—
<i>Prochlorococcus marinus</i> MIT 9301	—	+	+	1	—	—	—
<i>Prochlorococcus marinus</i> MIT 9303	—	+	+	—	—	—	—
<i>Prochlorococcus marinus</i> MIT 9312	—	+	+	1	—	—	—
<i>Prochlorococcus marinus</i> MIT 9313	—	+	+	—	—	—	—
<i>Prochlorococcus marinus</i> MIT 9515	—	—	+	1	—	—	—
<i>Prochlorococcus marinus</i> NATL1A	—	—	+	1	—	—	—
<i>Prochlorococcus marinus</i> NATL2A	—	—	+	1	—	—	—
<i>Prochlorococcus marinus</i> SS120°	—	+	+	1	—	—	—
<i>Prochlorococcus marinus</i> MED4	—	+	+	1	+	—	—

MGS: methylglyoxal synthase.

GlxI: glyoxalase I.

GlxII: glyoxalase II.

AkR: aldo/keto reductase.

Fdh1: glutathione-dependent formaldehyde dehydrogenase.

Fdh2: S-formylglutathione hydrolase.

S3: syntenic or genomic cluster fdh1–fdh2.

\*Presence of one gene between fdh1 and fdh2 in the reverse direction.



#### **4. GLUTATHIONE PROTECTS CELLS AGAINST CHEMICAL AND OXIDATIVE STRESSES VIA THE GLUTATHIONE TRANSFERASE SYSTEM**

Glutathione transferases (GSTs) constitute a protein superfamily that plays a key role in the detoxification of chemical and oxidative stresses in aerobic prokaryotes and eukaryotes, but not in anaerobic bacteria or in Archaea lacking GSH (Allocati, Federici, Masulli, & Di Ilio, 2009). The GST enzymes catalyse nucleophilic attack by GSH on the electrophilic groups of a wide range of hydrophobic toxic compounds, thus promoting their biodegradation and/or excretion from the cell. All known crystallized cytosolic GSTs are dimeric proteins, where each subunit is composed of two domains. The N-terminal domain binds GSH, whereas the C-terminal part makes most contacts with the electrophilic substrate. Eukaryotic and bacterial organisms have multiple GST genes of widely divergent sequences and in some cases unknown function. Four different classes of canonical GSTs, beta, chi, theta and zeta, have been identified in aerobic bacteria. In addition to the well-known beta-class GST, characterized by the presence of a cysteine residue at the GSH-binding site and a well-conserved overall structure, *E. coli* possesses six GST homologues. Two of them, YfcF and YfcG, exhibit GST- and GSH-dependent peroxidase activities and are involved in the defence against oxidative stress.

Cyanobacteria might have been the first organism to harbour GSTs (Wikteliu & Stenberg, 2007) because GSH and GSH-dependent enzymes are regarded as dating back to the evolution of an oxygen-containing atmosphere generated by cyanobacteria (Schopf, 2011). Consistently, all known cyanobacterial genomes do contain various numbers (2–16) of genes annotated as GSTs. The  $N_2$ -fixing filamentous cyanobacteria and the symbiotic *Acaryochloris* strain being the ones with the larger number of GST genes (more than 10). Two cyanobacterial GSTs, originating from the strains *Thermosynechococcus elongatus* BP-1 and *Synechococcus elongatus* PCC 6301, were studied and found to be belonged to the chi class of GSTs, though they lack cysteines completely (Wikteliu & Stenberg, 2007).



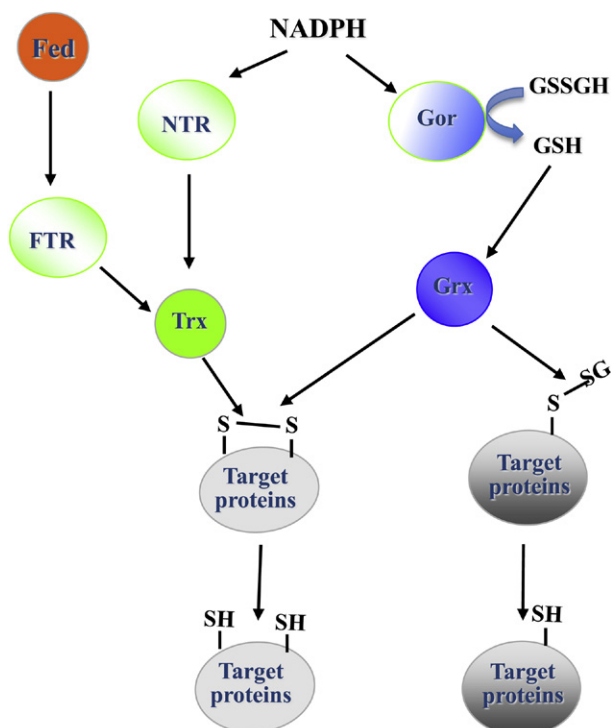
#### **5. GLUTATHIONE MAINTAINS THE REDOX HOMEOSTASIS OF PROTEIN THIOLS VIA THE GLUTAREDOXIN SYSTEM**

ROS can generate two types of disulfides depending whether they link two cysteinyl residues from the same or different proteins (protein–S–S–protein) or from a protein and a GSH molecule (protein–S–SG mix

disulfide, also termed glutathionylation). Many proteomics studies have shown that a large number of proteins from bacteria, cyanobacteria (Marteyn, Sakr, Chauvat and Cassier-Chauvat unpublished results), plants and human are glutathionylated under artificially imposed oxidative stress (Dalle-Donne et al., 2009; Zaffagnini, Bedhomme, Lemaire, & Trost, 2012). These proteins are involved in diverse processes, including photosynthesis, oxidative stress responses, protein folding, amino acid biosynthesis, lipid metabolism, translation, ATP metabolism, and cytoskeletal arrangements. In *Chlamydomonas reinhardtii* and/or plants, these glutathionylated proteins are, namely, glutathione S-transferase (GST); cytosolic triose phosphate isomerase and chloroplastic fructose-1,6-bisphosphate aldolase; PRX2 the thiol-dependent peroxidases; GAPDH; Trxf; ICL (iso-citrate lyase) (Zaffagnini, Bedhomme, Marchand et al., 2012). The glutathionylation process can protect the protein cysteines against irreversible oxidation, allowing them to be reduced back to their native states by Grxs when the organism has escaped from the oxidative environment (Zaffagnini, Bedhomme, Marchand et al., 2012). In addition, the glutathionylation/deglutathionylation process can also mediate the redox regulation of enzyme activity (activation or deactivation) (Dalle-Donne et al., 2009; Zaffagnini, Bedhomme, Lemaire et al., 2012).

The disulfide structures can be repaired (Fig. 5.3) by the small enzymes thioredoxins (Trxs) and glutaredoxins (Grxs), which share a similar three-dimensional structure known as the thioredoxin fold (Berndt et al., 2008). In the thioredoxin system, NADPH transfers its electrons to the thioredoxin reductase enzyme (NTR, a flavoenzyme) to reduce Trxs, which use two redox-active cysteines in a conserved CxxC active site motif to reduce protein-S-S-protein disulfides (Berndt et al., 2008; Lillig et al., 2008). In the glutaredoxin system, which preferentially reduces protein-S-SG mixed disulfides (Lillig et al., 2008; Rouhier et al., 2008; Zaffagnini, Bedhomme, Marchand et al., 2012), NADPH transfers its electrons to the glutathione reductase enzyme (GR, a flavoenzyme) present in many, but not all (Marteyn et al., 2009), organisms (Table 5.2) to reduce GSH and subsequently Grxs. In addition, the Grx enzymes can also be reduced by NTR (Marteyn et al., 2009; Couturier et al., 2009), or by the ferredoxin-dependent thioredoxin reductase (FTR) enzyme that receives its electrons from photosynthesis (Zaffagnini, Bedhomme, Lemaire et al., 2012; Zaffagnini, Bedhomme, Marchand et al., 2012).

The analysis of the specificity and redundancy of the Grx and Trx enzymes is difficult in plants because they possess multiple Grxs and Trxs, respectively, 31 and 19 in *A. thaliana* (Michelet et al., 2006). By contrast, cyanobacteria possess a smaller number of *trx* and *grx* genes (Table 5.2). For instance, the model strain *Synechocystis* PCC 6803 has only four Trxs and three Grxs.



**Figure 5.3 The disulfide reduction pathways.** Fed: Ferredoxin, FTR: Ferredoxin dependant thioredoxin reductase, GR: Glutathione reductase, Grx: Glutaredoxin, GSH: Glutathione (reduced form), GSSG: Glutathione disulfide (oxidized glutathione), NTR: NADPH-dependant thioredoxin reductase, -S-SG: Protein-glutathione mixed disulfide, Trx: Thioredoxin.

The four Trxs of *Synechocystis* PCC 6803 belong to four different Trx types encoded by the genes *trxA* (m-type; slr0623), *trxB* (x-type; slr1139), *trxC* (slr11057) and *trxQ* (y-type; slr0233). Both *trxA* and *trxC* are essential for normal photoautotrophic growth (their attempted inactivation invariably led to a heteroploid strain harbouring both wild-type and mutant copies of the chromosome, with and without the studied *trx* genes (Florencio, Perez-Perez, Lopez-Maury, Mata-Cabana, & Lindahl, 2006). By contrast, fully segregated *trxB*-less and *trxQ*-less mutants were obtained indicating that both TrxB and TrxQ are dispensable to cell growth. Furthermore, a strain containing a mutated version of TrxB (TrxB<sub>C34S</sub>) was used to identify the potential in vivo TrxB targets by a proteomic analysis (Perez-Perez, Martin-Figueroa, & Florencio, 2009). These TrxB targets are involved in protein synthesis (ribosomal proteins), metabolism of amino acids (glutamine synthase, cysteine synthase) and sugars (fructose biphosphate aldolase,

fructose-1,6-bisphosphatase, pyruvate dehydrogenase, glyceraldehyde-3-phosphate dehydrogenase, phosphoribulokinase, ribulose biphosphate carboxylase, GDP-mannose 4,6-dehydratase, transketolase), and protection against oxidative stress (1-Cys peroxiredoxin).

All cyanobacteria possess two types of Grx enzymes harbouring either a CxxC- or a CGFS-type redox sites (Table 5.2). The CxxC Grxs have been studied in various prokaryotes and eukaryotes organisms (Herrero, Belli, & Casa, 2010; Lillig *et al.*, 2008; Masip *et al.*, 2006; Rouhier *et al.*, 2008; ). They catalyse the reduction of both protein disulfides (protein-S-S-protein) and glutathione mixed disulfide (protein-S-SG) by a dithiol or a monothiol mechanism. In the dithiol mechanism, the N-terminal active site cysteine resolves the disulfide, or the protein-S-SG mixed disulfide of the glutathionylated proteins. Then, the oxidized cysteine of the Grx enzyme is reduced by its second active site cysteine, itself subsequently reduced by the NTR or FTR enzymes (Zaffagnini, Bedhomme, Lemaire *et al.*, 2012). In the monothiol mechanism, after its oxidation, the Grx active site cysteine is subsequently reduced by GSH. Several laboratories have analysed the two CxxC Grxs of the model cyanobacterium *Synechocystis* PCC 6803, Grx1 (Slr1562 in Cyanobase) and Grx2 (Ssr2061), which share the conserved CPFC redox motif driving the thiol oxidoreductase activity (Li, Huang, Yang, Liu, & Wu, 2005; Marteyn *et al.*, 2009). Grx2 serves as an electron donor to the arsenate reductase enzyme that catalyses the detoxification of arsenate that is frequently encountered in cyanobacterial biotopes (Kim *et al.*, 2012). Furthermore, a Grx2 monothiol mutant was used to capture 42 prey proteins operating in carbon metabolism, protein synthesis and folding, light harvesting and protection against oxidative stress (Li *et al.*, 2007). Interestingly, 13 of these Grx2 target proteins, including the catalase-peroxidase and a peroxyredoxin, were also captured by a Trx monothiol mutant (Florencio *et al.*, 2006), emphasizing on the cross-talk between the Grx and Trx enzymes. Also interestingly, we found that both Grx1 and Grx2 operate in a novel integrative redox pathway, NTR-Grx1-Grx2-Fed7 (Fed7 stands for ferredoxin 7), which protects *Synechocystis* PCC 6803 against selenate, another frequent pollutant (Marteyn *et al.*, 2009). Furthermore, we showed that under selenate stress, Grx1 and Grx2 assemble under the form of a heterodimer. It will be interesting to investigate further the influence of environmental conditions on the possible formation of homo/heterodimers of Grx1 and Grx2 since their counterpart in noncyanobacterial organisms can form dimers bridged by a GSH-ligated 2Fe-2S cluster, which regulates the thiol oxidoreductase

activity and operates in oxidative-stress sensing (Lillig et al., 2008; Rouhier, Couturier, Johnson, & Jacquot, 2010).

We have initiated the analysis of the single CGFS-type Grx, which is present in all cyanobacteria (Table 5.2). We showed that the enzyme (Grx3) of *Synechocystis* PCC 6803 forms a homodimer bridged by a GSH-ligated 2Fe–2S cluster, and that this feature is conserved in the orthologous enzymes from bacteria, cyanobacteria, yeast, plants and mammals (Iwema et al., 2009; Picciocchi, Saguez, Boussac, Cassier-Chauvat, & Chauvat, 2007). This finding was confirmed by several groups (Herrero et al., 2010; Lillig et al., 2008; Rouhier et al., 2010), and one of these Grxs was showed to be able to transfer its GSH-anchored 2Fe–2S centre to the apoform of a 2Fe–2S ferredoxin (Rouhier et al., 2010). These results are important as iron–sulphur centres play a crucial role in the electron transfers associated with photosynthesis, respiration and carbon metabolism (Lillig et al., 2008; Rouhier et al., 2010). CGFS Grxs can also interact with the widely conserved BolA protein family. In yeast, it has been shown that the interactions between Grx3 or Grx4 with the BolA-like protein Fra2 operate in iron regulation mediated by the Aft1 and Aft2 transcriptional regulators. These heterodimeric complex BolA–Grx possesses a (2Fe–2S) cluster ligated by a histidine from Fra2, a cysteine from either Grx3 or Grx4, and the cysteine from GSH. Similar findings were observed with the corresponding human proteins (Li, Mapolelo, Randeniya, Johnson, & Outten, 2012), thereby establishing the ubiquitous CGFS Grxs and BolA-like proteins as a novel type of 2Fe–2S cluster binding regulatory complex. Further investigations are required to determine whether the BolA-like and CGFS Grx proteins of cyanobacteria can also assemble as a heterodimer bridged by a GSH-ligated 2Fe–2S cluster operating in iron regulation which is vital to cyanobacteria (Houot et al., 2007; Shcolnick et al., 2009).



## 6. CONCLUDING REMARKS

It is important to analyse the defences against oxidative stress in cyanobacteria because they are the organisms that developed most of these mechanisms as a crucial necessity to cope with the production of ROS by their active photoautotrophic metabolism, which supports a large part of the biosphere and has valuable biotechnological potentials. Furthermore, many of the effective anti-oxidant processes that likely emerged in cyanobacteria have been conserved, and complexified in higher plants and mammals.

In the past few years, significant progress has been made in the identification of ROS-scavenging and detoxification processes in cyanobacteria, unravelling the crucial and pleiotropic role of glutathione and glutathione-dependent enzymes, such as the glutaredoxin (Grxs) enzymes, which control the redox state of cellular thiols; the glyoxalases (Glxs), which detoxify the toxic metabolite methylglyoxal; and the glutathione transferases (GSTs), which detoxify many xenobiotics. The selectivity-redundancy of these enzyme families need to be thoroughly investigated to determine what range of oxidants (GSTs) or oxidized proteins (Grxs) they can detoxify (Gsts) or repair (Grxs), and in which subcellular compartment they act. Another important challenge in the near future will be to analyse how redox regulation of proteins by glutathionylation and deglutathionylation via the Grxs enzymes affects global gene expression and metabolism in cells growing in various environmental conditions or facing oxidative challenges. These studies will be facilitated by the recent breakthroughs in genome sequencing and comparative genomics, which enable genome-based reconstruction of an organism's metabolism, as well as the powerful genetics of various model strains. Indeed, the bioinformatic method of "prediction by analogy" has its limits. Until we do the experiments, we may mispredict the real function or properties of a gene product (Bender, 2011; Domain *et al.*, 2004; Figge *et al.*, 2001).

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# A Genomic View of Secondary Metabolite Production in Cyanobacteria

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## Abstract

Cyanobacteria produce a wide range of secondary metabolites that are very diverse in chemical structure. These metabolites show also very diverse biological activities, including cytotoxicity, neurotoxicity, dermatotoxicity, and inhibition of proteases. The cyanotoxins that are harmful to animals, including humans, are essentially produced by freshwater cyanobacteria, while marine and terrestrial cyanobacteria produce some metabolites that are promising new drugs. Between 2001 and 2010, many biosyntheses of cyanobacterial secondary metabolites have been deciphered at the genetic and biochemical level. Thanks to the advent of genomic data on cyanobacterial genomes and to new powerful bioinformatic tools, about 30 clusters of genes responsible for the production of cyanobacterial secondary metabolites have been identified. The biosyntheses have also been studied *in vitro*, in certain cases. For instance, among the cyanotoxins, the microcystin, the cylindrospermopsin, the saxitoxin and the anatoxin-a biosyntheses have been elucidated. Almost all cyanobacterial secondary metabolites are the products of polyketide synthases, nonribosomal peptide synthases or hybrid thereof. However, ribosomal peptides are also produced by cyanobacteria, like the cyanobactins and recent genome mining data suggest that these metabolites are more represented than first thought in cyanobacteria. This review gives an overview of the connections between cyanobacterial secondary metabolites and their biosynthetic genes, with emphasis on the most significant cases like the cyanotoxins, sunscreens, alkanes and terpenes.



## ABBREVIATIONS

**A** adenylation domain

**ACP** acyl carrier protein

**Adda** (2S, 3S, 8S, 9S)-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid

**Ahda** 3-amino-2-hydroxy-decanoate

**Ahoa** (2S, 3R, 5R)-amino-2,5-dihydroxy-8-phenyloctanoate

**Ahp** 3-amino-6-hydroxy-2-piperidone

**AMT** aminotransferase domain

**AT** acyltransferase domain

**C** condensation domain

**Choi** 2-carboxy-6-hydroxyoctahydroindole  
**CM** C-methyltransferase domain  
**DH** dehydratase domain  
**E** epimerase domain  
**EP** epimerase domain  
**ER** enoylreductase domain  
**KR** ketoreductase domain  
**KS** ketosynthase domain  
**MIB** 2-methylisoborneol  
**MT** methyltransferase domain  
**NRP** nonribosomal peptide  
**NRPS** nonribosomal peptide synthase  
**OM** O-methyltransferase domain  
**PCP** peptidyl carrier protein  
**PK** polyketide  
**PK/NRP** polyketide/nonribosomal peptide hybrid  
**PKS** polyketide synthase  
**PKS/NRPS** polyketide synthase/nonribosomal peptide synthase hybrid  
**RP** ribosomal peptide  
**TE** thioesterase domain.



## 1. INTRODUCTION

Natural products, including microbial secondary metabolites, have played a prominent role in the development of drugs to treat infections, cancers and many other diseases (Newman & Cragg, 2012). They have also inspired synthetic chemists because of the tremendous chemical diversity encountered in these metabolites produced by living organisms, and because of the interest in modifying the structure of natural products to obtain new molecules with superior bioactivities. It is thus not surprising that this scientific field is still very active (Walsh & Fischbach, 2010).

Historically, natural products were isolated from plants, marine organisms and microorganisms, and their chemical structure was then elucidated, and their biological activity was screened. This traditional approach is still used and is still necessary to find new bioactive molecules and to prove the structure of the isolated metabolite. However, the natural product field made an important step when the genes coding for the enzymes involved in the biosynthesis of the natural antibiotic erythromycin, a macrocyclic polyketide (PK), were identified and sequenced (Cortes, Haydock, Roberts, Bevitt, & Leadlay, 1990; Donadio, Staver, McAlpine, Swanson, & Katz, 1991). The core structure of this antibiotic is produced by three giant polyketide synthases (PKSs) that are modular with each module responsible for the introduction of two carbons from acetate.



The modules consist of different domains, each catalysing a particular reaction that modifies the acetate unit leading to a ketone, an alcohol, an alkene or a completely reduced carbon. The metabolite, tethered to the PKSs by a thioester bond, is thus built on these megasynthases in a sequential manner just like on an assembly line: each module adds one acetate unit and modifies it using the same set of domains (Staunton & Weissman, 2001). This assembly line enzymology has also been found in another class of synthases, the nonribosomal synthases (NRPSs), responsible for the biosynthesis of peptidic secondary metabolites (Sieber & Marahiel, 2005). In these NRPSs, the building blocks are amino acids, either proteinogenic or not, and each module adds sequentially a residue and forms a peptide bond to yield the final peptide. In both class of enzymes, the PKSs and NRPSs, the different modules are usually arranged in line and there is a co-linearity between the amino acid sequence of the synthase, from the N-terminal to the C-terminal end, and the reaction sequence that yields to the full metabolite, the so-called co-linearity rule. The reader is referred to excellent and authoritative reviews covering this fascinating field of enzymology (Fischbach & Walsh, 2006; Staunton & Weissman, 2001).

With the advent of huge amount of microbial genomic data (more than a thousand of microbial genomes have so far been sequenced), it appears that (1) the genes responsible for the biosynthesis of microbial secondary metabolites are usually clustered in the genome of the microorganism; (2) the domains of the PKSs and NRPSs are conserved and their function and specificity can be predicted using bioinformatic tools; and (3) because of the co-linearity rule, though not always respected, it has been possible to predict, yet with still some uncertainty, the structure of the metabolite produced by these synthases. The reverse prediction is also possible in certain cases, again with some uncertainty, that is, the possibility to predict the biosynthetic genes from the structure of the metabolite. Thus, genome mining has become an exciting new way that is complementary to the traditional extraction/identification strategy, to discover novel secondary metabolites (Challis, 2008).

In this review, the authors have tried to give the reader an up-to-date vision of the connections between secondary metabolites from cyanobacteria and their biosynthetic genes in a growing postgenomic era.

## 2. THE CYANOBACTERIAL SECONDARY METABOLITES

Cyanobacteria represent a large group of bacteria that have evolved for a very long period of time ( $3.5 \times 10^9$  years) and this is probably the reason why these bacteria are very diverse in terms of morphology and

genetics (or genomes). They also produce a wide range of secondary metabolites that are very diverse in chemical structure: peptides, PKs, peptide and PK hybrids, alkanes, terpenes, etc. (Kehr, Gatte Picchi, & Dittmann, 2011; Nunnery, Mevers, & Gerwick, 2010). Again, this chemical diversity probably reflects the long evolution of cyanobacteria and their adaptation to diverse ecosystems, growth conditions and competitors or predators. For instance, at least 800 different secondary metabolites have so far been identified in marine cyanobacteria and it is believed that this represents only a small fraction of the natural product repertoire (Jones et al., 2010).

The functions of cyanobacterial secondary metabolites, apart from the sunscreens, are usually unknown but it is expected that the production of a specific metabolite has given, or gives, some advantage to the producer in a complex ecosystem. It has also been proposed that these molecules might be communication molecules although there is no firm experimental data on this issue. Nevertheless, some of the cyanobacterial secondary metabolites are toxic to higher animals, including humans, and have thus been called cyanotoxins (van Apeldoorn, van Egmond, Speijers, & Bakker, 2007). Because the presence of these cyanotoxins in the environment poses a threat to animal and human health, they have been the primary focus of the research on cyanobacterial secondary metabolites. However, cyanobacteria also produce secondary metabolites that are potential therapeutic drugs, such as cryptophycins, and there is a constant interest in discovering new natural products from cyanobacteria as potential drug candidates (Nunnery et al., 2010).

Another interesting aspect of the research concerning cyanobacterial secondary metabolites concerns the production of metabolites that can be used as biofuels. Because these photosynthetic bacteria use carbon dioxide as carbon source and produce alkanes, alkenes, alcohol, esters or terpenes and related compounds, there is a growing interest in using these microorganisms as sustainable factories for producing environmentally friendly fuels (Wackett, 2011).

Not all cyanobacteria are prolific producers of secondary metabolites. The unicellular cyanobacteria, belonging to genera such as *Prochlorococcus* or *Synechococcus*, usually do not produce secondary metabolites while filamentous (*Anabaena*, *Planktothrix*, or *Lyngbya*) or colonial (*Microcystis*) cyanobacteria produce diverse secondary metabolites. Interestingly, the genome size of filamentous cyanobacteria is larger by 5–6 Mb than that of unicellular species as if the genome of unicellular cyanobacteria has only space for primary metabolism. As noted above, filamentous marine cyanobacteria produce a

wide range of secondary metabolites, and they are considered as a potential source of molecules with therapeutic activities. Freshwater cyanobacteria are better known as toxin producers although they certainly produce valuable compounds in terms of therapeutic potential. There is a growing interest in studying terrestrial or symbiotic cyanobacteria, although not much is known on these microorganisms, because they produce diverse secondary metabolites with interesting biological activities, such as the cryptophycins.



### **3. OVERVIEW OF SECONDARY METABOLITES FROM CYANOBACTERIA FOR WHICH BIOSYNTHETIC GENES HAVE BEEN IDENTIFIED**

While identification of secondary metabolites from cyanobacteria has a long history, the identification of the biosynthetic genes responsible for the production of secondary metabolites, in these microorganisms, is relatively recent (after year 2000). We have summarized in [Table 6.1](#) essential data concerning the cyanobacterial secondary metabolites for which the biosynthetic genes have been identified. Among all the cyanobacterial metabolites isolated (probably over a thousand), we have only found about 30 cases for which the biosynthetic genes have been identified. The main reason for this low number of clusters discovered is that the identification of these genes is highly dependent on genomic data on the producer. In fact, there are about 100 cyanobacterial genomes so far sequenced and the majority of them are from unicellular species that do not produce secondary metabolites ([Hess, 2011](#)). Finally, the fact that the genetic manipulations of cyanobacteria are difficult has also probably hampered the discovery of biosynthetic genes implicated in secondary metabolism.

#### **3.1. The Metabolites**

It is not easy to classify the secondary metabolites from cyanobacteria because they are very diverse in structure. We have followed a simple classification based on the origin of the secondary metabolite: first, the cyanotoxins from freshwater cyanobacteria, followed by other metabolites from freshwater cyanobacteria, and then metabolites from terrestrial cyanobacteria, followed by metabolites from marine cyanobacteria and finally, ribosomal peptides (RPs) and other metabolites.

As already noted, the structural diversity in the metabolites described in this review is very large. It is also interesting to note that for each class of metabolite, there are almost always variants that have been detected.

**Table 6.1** Cyanobacterial secondary metabolites with known biosynthetic gene cluster

Metabolite*	Producing strain	Metabolite structural class	Biological activity	Cluster (size)	References
<b>Fresh water cyanotoxins</b>					
Microcystins	<i>Microcystis aeruginosa</i> PCC 7806	PK/NRP	Hepatotoxin	<i>mcy</i> (55 kb)	(Tillett et al., 2000)
	<i>Microcystis aeruginosa</i> NIES 843				(Kaneko et al., 2007)
	<i>Planktothrix rubescens</i> NIVA CYA 98				(Rounge et al., 2009)
	<i>Anabaena</i> sp. 90				(Rouhiainen et al., 2004)
	<i>Planktothrix agardhii</i> NIVA CYA 126/8				(Christiansen et al., 2003)
Nodularins	<i>Nodularia spumigena</i> NSOR 10	PK/NRP	Hepatotoxin	<i>nda</i> (48 kb)	(Moffitt & Neilan, 2004)
Anatoxin-a/homo-anatoxin-a	<i>Oscillatoria</i> sp. PCC 6506	PK/NRP	Neurotoxin	<i>ana</i> (29 kb)	(Méjean et al., 2009)
	<i>Anabaena</i> sp. 37				(Rantala-Ylinen et al., 2011)
Saxitoxins	<i>Cylindrospermopsis raciborskii</i> T3	Alkaloid	Neurotoxin	<i>sxt</i> (35 kb)	(Kellmann et al., 2008)
	<i>Anabaena circinalis</i> 131C				(Mihali et al., 2009)
	<i>Aphanizomenon</i> NH5				(Mihali et al., 2009)
	<i>Raphidiopsis brookii</i> D9				(Stucken et al., 2010)
	<i>Lyngbya wollei</i>				(Murray et al., 2011)
Cylindrospermopsins	<i>Cylindrospermopsis raciborskii</i> AWT 205	PK/NRP	Cytotoxin	<i>cyr</i> (43 kb)	(Mihali et al., 2008)
	<i>Aphanizomenon</i> sp. 10E6				(Stuken & Jakobsen, 2010)
	<i>Oscillatoria</i> sp. PCC 6506				(Mazmouz et al., 2010)
	<i>Cylindrospermopsis raciborskii</i> CS 505				(Stucken et al., 2010)
	<i>Raphidiopsis curvata</i> CHAB1150				(Jiang et al., 2012)

Continued

**Table 6.1** Cyanobacterial secondary metabolites with known biosynthetic gene cluster—cont'd

Metabolite*	Producing strain	Metabolite structural class	Biological activity	Cluster (size)	References
<b>Other fresh water metabolites</b>					
<b>Aeruginosins</b>		PK/NRP	Protease inhibitor	<i>aer</i> (34 kb)	
Aeruginosides 126	<i>Planktothrix agardhii</i> NIVA CYA 126/8				(Ishida et al., 2007)
Aeruginosin 686	<i>Microcystis aeruginosa</i> PCC 7806				(Ishida et al., 2009)
Aeruginosin 98	<i>Microcystis aeruginosa</i> NIES 98				(Ishida et al., 2009)
Aeruginosin 102	<i>Microcystis aeruginosa</i> NIES 843				(Ishida et al., 2009)
Aeruginosin A	<i>Planktothrix rubescens</i> NIVA CYA 98				(Rounge et al., 2009)
Microginins	<i>Planktothrix rubescens</i> NIVA CYA 98	PK/NRP	Angiotensin converting enzyme inhibitor	<i>mic</i> (23 kb)	(Rounge et al., 2009)
<b>Cyanopeptolins</b>		NRP	Protease inhibitor		
Oscillapeptin G	<i>Planktothrix rubescens</i> NIVA CYA 98			<i>oci</i> (35 kb)	(Rounge et al., 2009)
Cyanopeptolin 1138	<i>Planktothrix agardhii</i> NIVA CYA 116			<i>oci</i> (30 kb)	(Rounge et al., 2007)
Cyanopeptolin 984	<i>Microcystis cf. wesenbergii</i> NIVA CYA 172/5			<i>mcn</i> (30 kb)	(Tooming-Klunderud, Rohrlack, Shalchian-Tabrizi, Kristensen, & Jakobsen, 2007)

Anabaenopeptilides <b>Anabaenopeptins</b>	<i>Anabaena</i> sp. 90	NRP	Protease inhibitor	<i>apd</i> (28 kb)	(Rouhiainen et al., 2000)
AnabaenopeptinA, B, C	<i>Anabaena</i> sp. 90			<i>apt</i> (32 kb)	(Rouhiainen et al., 2010)
Nodulaeptin B, C	<i>Nodularia spumigena</i> CCY9414			<i>apt</i> (26 kb)	(Rouhiainen et al., 2010)
Anabaenopeptin NZ 857	<i>Nostoc punctiforme</i> PCC 73102			<i>apt</i> (26 kb)	(Rouhiainen et al., 2010)
Oscillamid Y	<i>Planktothrix rubescens</i> NIVA CYA 98			<i>ana</i> (24 kb)	(Rounge et al., 2009)
Anabaenopeptin 908, 915	<i>Planktothrix agardhii</i> NIVA CYA 126/8			<i>apn</i> (24 kb)	(Christiansen et al., 2011)
<b>Terrestrial metabolites</b>					
Cryptophycins	<i>Nostoc</i> ATCC 53789	PK/NRP	Cytotoxic	<i>crp</i> (40 kb)	(Magarvey et al., 2006)
Nostophycins	<i>Nostoc</i> sp. strain 152	PK/NRP	Cytotoxic	<i>nnp</i> (45 kb)	(Fewer et al., 2011)
Nostocyclopeptides	<i>Nostoc</i> ATCC 53789	NRP		<i>nep</i> (33 kb)	(Becker et al., 2004)
Nostopeptolides	<i>Nostoc</i> sp. GSV224	PK/NRP		<i>nos</i> (40 kb)	(Hoffmann et al., 2003)
<b>Marine metabolites</b>					
Curacins	<i>Lyngbya majuscula</i> 3L	PK/NRP	Cytotoxin	<i>cur</i> (63 kb)	(Chang et al., 2004)
Hectochlorin	<i>Lyngbya majuscula</i> JHB	PK/NRP	Cytotoxin	<i>hct</i> (38 kb)	(Ramaswamy, Sorrels, & Gerwick, 2007)
Apratoxins	<i>Lyngbya bouillonii</i>	PK/NRP	Cytotoxin	<i>apr</i> (58 kb)	(Grindberg et al., 2011)
Jamaicamides	<i>Lyngbya majuscula</i> JHB	PK/NRP	Neurotoxin	<i>jam</i> (57 kb)	(Edwards et al., 2004)
Lyngbyatoxins	<i>Lyngbya majuscula</i>	NRP	Dermatotoxin	<i>ltx</i> (11 kb)	(Edwards & Gerwick, 2004)
Barbamides	<i>Lyngbya majuscula</i> 3L	PK/NRP		<i>bar</i> (26 kb)	(Chang et al., 2002)

Continued

**Table 6.1** Cyanobacterial secondary metabolites with known biosynthetic gene cluster—cont'd

Metabolite*	Producing strain	Metabolite structural class	Biological activity	Cluster (size)	References
<b>Ribosomal metabolites</b>					
<b>Cyanobactins<sup>†</sup></b>		Modified peptides	Various activities		
Microcyclamide	<i>Microcystis aeruginosa</i> PCC 7806			<i>mca</i> (11 kb)	(Ziemert, Ishida, Liaimer, et al., 2008; Ziemert, Ishida, Quillardet, et al., 2008)
Patellamide	<i>Prochloron</i> spp. <sup>‡</sup>			<i>pat</i> (10 kb)	(Schmidt et al., 2005)
<b>Microviridins</b>		Modified peptides	Protease inhibitor		
Microviridin B, B2, B3	<i>Microcystis aeruginosa</i> NIES 298			<i>mdn</i> (9 kb)	(Ziemert, Ishida, Liaimer, et al., 2008; Ziemert, Ishida, Quillardet, et al., 2008)
Microviridin J	<i>Microcystis aeruginosa</i> UOWOCC			<i>mdn</i> (9 kb)	(Ziemert, Ishida, Liaimer, et al., 2008; Ziemert, Ishida, Quillardet, et al., 2008)
Microviridin K	<i>Planktothrix agardhii</i> NIVA CYA 126/8			<i>mvd</i> (7 kb)	(Philmus et al., 2008)
Putative microviridin	<i>Planktothrix rubescens</i> NIVA CYA 98			<i>mdn</i> (7 kb)	(Rounge et al., 2009)

**Others**

Scytonemin	<i>Nostoc punctiforme</i> ATCC 29133	Amino acid derived	Sunscreen	<i>scy</i> (6 kb)	(Balskus & Walsh, 2009; Soule et al., 2007)
Shinorin	<i>Anabaena variabilis</i> ATCC 29413	Amino acid derived	Sunscreen	<i>orf</i> 3858–3855 (6 kb)	(Balskus & Walsh, 2010)
Mycosporine-glycine	<i>Nostoc punctiforme</i> ATCC 29133	Amino acid derived	Sunscreen	<i>mys</i> (4.5 kb)	(Gao & Garcia-Pichel, 2011b)
Alkanes	<i>Synechococcus elongatus</i> PCC 7942	Fatty acid derived		<i>orf</i> 1593–1594 (2 kb)	(Schirmer et al., 2010)
Alkenes	<i>Synechococcus</i> sp. PCC 7002	Fatty acid derived		<i>ols</i> (8 kb)	(Mendez-Perez et al., 2011)
Geosmin	<i>Nostoc punctiforme</i> PCC 73102	Terpene	Smelly molecule	Npun020003620 (2 kb)	(Giglio, Jiang, Saint, Cane, & Monis, 2008)
Methylisoborneol	<i>Pseudanabaena</i> sp. dqh15	Terpene	Smelly molecule	<i>mtf</i> , <i>mic</i> (2 kb)	(Wang, Fewer, et al., 2011; Wang, Xu, et al., 2011)
	<i>Planktothricoides raciborskii</i> CHAB3331				(Wang, Fewer, et al., 2011; Wang, Xu, et al., 2011)

\*The structure of the metabolites are shown in Fig. 6.1–6.17. Fig. 6.17 Shows the structure of the metabolites that are not discussed in details in the text in section 4.

†Only two examples are given but there are a large number of cyanobactins identified (more than a hundred).

‡Symbiotic cyanobacteria of the ascidia *Lissoclinum patella*.



The origin of these structural variations is a very interesting aspect of the research in this field and will be discussed below.

The majority of the metabolites described in Table 6.1 are products of PKS, NRPS or more frequently of PKS/NRPS hybrids, but several recent studies suggest that RPs are much more represented than believed in the cyanobacteria (Li et al., 2010; Velasquez & van der Donk, 2011; Wang, Fewer, et al., 2011; Wang, Xu, et al., 2011). Thus, the data reported in Table 6.1 are probably biased. Nevertheless, it is quite interesting to note that PKSs and NRPSs are capable to produce very different molecules even though their basic functions supported by the individual domains are conserved, particularly for the PKSs. Not surprisingly, the biological activities of the secondary metabolites are very diverse.

### 3.2. The Producing Strains

We have also indicated in Table 6.1 the producers, in which the biosynthetic genes have been sequenced. It can be seen that many genera are represented, but as indicated earlier in this review, the producers are mostly filamentous or colonial cyanobacteria. As expected, some cyanobacteria produce several secondary metabolites. For instance, the strains *Planktothrix agardhii* NIVA-CYA 126/8 and *Planktothrix rubescens* NIVA-CYA 98 are cited four and six times, respectively in Table 6.1. These two strains have been well characterized and their genome has been sequenced, and they produce several secondary metabolites. This is a good illustration of the capacity of cyanobacteria to produce many secondary metabolites at the same time, and it is expected that this is the case for many other cyanobacteria. It is also clear from Table 6.1 that metabolites from freshwater cyanobacteria are produced by different genera (see, for instance, cylindrospermopsin producers). This is very likely a result of horizontal gene transfer events as discussed below. Thus, a particular metabolite can be found in many different freshwater cyanobacteria. However, it seems that freshwater cyanobacteria and marine cyanobacteria do not produce the same set of secondary metabolites, except for the cyanobactins that have been found in either class of cyanobacteria (Donia & Schmidt, 2011). This situation might of course be a simple bias and it is possible that in the future, this apparent frontier show some leaks.

### 3.3. The Biosynthetic Clusters

The biosynthetic gene clusters identified are quite large, over 20 kb for PKs, NRPs, and PK/NRP hybrids and of course smaller (about 10 kb)

in the case of RPs since in these cases, the biosynthetic machinery is the ribosome. For the sunscreens, alkanes, alkenes and terpenes, the clusters are small, and for geosmin, a single enzyme is responsible for the biosynthesis. As illustrated in the following sections, the identification of the biosynthetic clusters followed the same scenario in many cases: the structure of the metabolites was known, feeding experiments using isotopically labelled precursors gave some clues concerning the biosynthesis (the incorporation of acetate reveals the involvement of PKSs, for instance) and a particular gene was then identified by using degenerative PCR amplifications. The link between the identified gene and the biosynthesis was then inferred from two different types of data: either the genetic inactivation of the identified gene abolished the production of the metabolite, an unambiguous and of course preferred experiment, or a correlation was found in a series of producers and nonproducers of the metabolite between the presence of the gene (genotype) and the production of the metabolite (phenotype). This latter indirect evidence has been used when genetic manipulation of the producer was not possible. Then, using either genomic libraries cloned in cosmids or genome sequence data, the cluster was identified and sequenced. In some interesting cases, the gene clusters were directly identified by genome mining, for example, in the case of alkane production (Schirmer, Rude, Li, Popova, & del Cardayre, 2010).

As noted above, genetic manipulation of cyanobacteria is not always possible and gene inactivation to prove the function of a specific cluster has only been described in a few cases. In the other cases, *in vitro* characterization of the biosynthetic enzymes afforded a firm experimental evidence for the function of the identified cluster. But in some cases, there is still no direct proof at all, except for the correlation between the biosynthetic pathway predicted from the bioinformatic analysis of the gene cluster and the biosynthesis imagined from the experimental data like isotopic feeding experiments, or from the structure of the metabolite. In the case of small cluster, like the cyanobactin gene clusters or the alkane gene cluster, heterologous expression in *Escherichia coli* of the entire cluster afforded an elegant proof for the function of the cluster. However, this type of experiment is not always possible due to the size of the clusters.

In some cases, several clusters responsible for the biosynthesis of the same metabolite have been sequenced, in different strains or genus, affording possible comparisons. These types of studies have been conducted in the case of the *mcy*, *stx*, *cyr*, *aer*, *ana* and cyanobactin gene clusters (see section 4

for specific case). The relevant information gathered from these studies are that horizontal gene transfer events are probably responsible for the presence of the same clusters in the genome of cyanobacteria of different genus. This is supported by the fact that transposase genes have been found next to or within the clusters of gene responsible for the biosynthesis of secondary metabolites. Other genetic events such as mutations, rearrangement of the genes, recombinations, deletions and insertions have been proposed to be responsible for the differences observed in the same cluster found in different cyanobacteria.

### 3.4. The Metabolite Biosyntheses

We have not described, in this review, all the biosynthetic schemes that have been derived from analysis of the clusters described in [Table 6.1](#). Instead, we have selected some representative examples that highlight the salient issues concerning the biosynthesis of cyanobacterial secondary metabolites. As noted above, the majority of the clusters so far described code for PKSs, NRPSs and mostly PKS/NRPS hybrids, but the reactions catalysed by these synthases show, in many cases, divergence from the standard PKSs and NRPSs enzymology. Indeed, the starters of PKSs are different from the standard acetyl or propionyl starter, and are very diverse in structure (in the cylindrospermopsin, anatoxin-a, and saxitoxin biosynthesis for instance). In the cyanobacterial PKSs, some domains show new reactions as the Mannich-type cyclization in the biosynthesis of anatoxin-a, or the numerous cyclization steps in the biosynthesis of cylindrospermopsin. The termination steps in these PKSs-mediated biosyntheses are frequently different from the standard case and involve hydrolysis, decarboxylation, or reduction. These differences from the standard PKS/NRPS reactions allow the formation of many various metabolites by the cyanobacterial producers. Another, general trait is that usually one cluster is responsible for the biosynthesis of several variants of one metabolite. The variations observed are either a change in amino acid residue, a methylation, a hydroxylation, a sulfatation, a halogenation, or a change in configuration at some carbons. These variations are either catalysed by tailoring enzymes or by some domains of the PKSs or the NRPSs that show a relaxed specificity. For instance, the variants observed in the microcystin family are generated by promiscuous adenylation domains or by mutations in these domains.

Not much is known concerning the regulation of the production of the cyanobacterial secondary metabolites. In only a few cases, the transcriptional

signals have been identified and the transcription regulation studied. It seems that light plays a central role in the transcription level in *mcy* or *cyr* operons for instance.

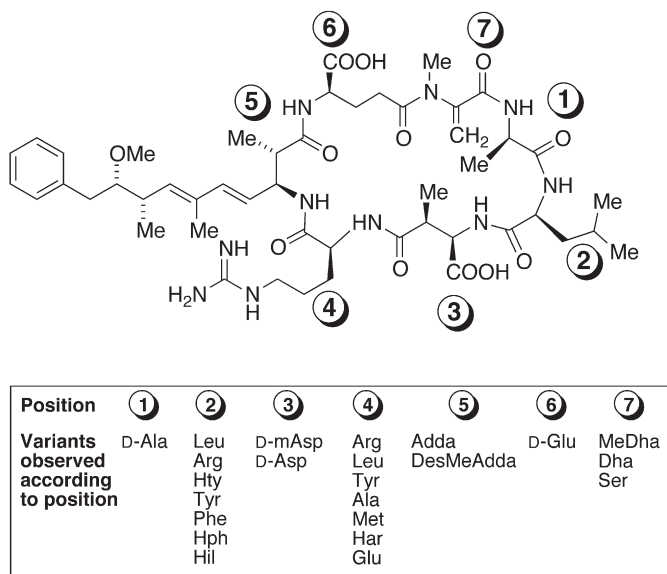


## 4. DESCRIPTION OF THE BIOSYNTHESIS AT THE GENOMIC AND BIOCHEMICAL LEVEL FOR SELECTED CYANOBACTERIAL SECONDARY METABOLITES

### 4.1. Cyanotoxins from Freshwater Cyanobacteria

#### 4.1.1. The hepatotoxins – microcystins and nodularins

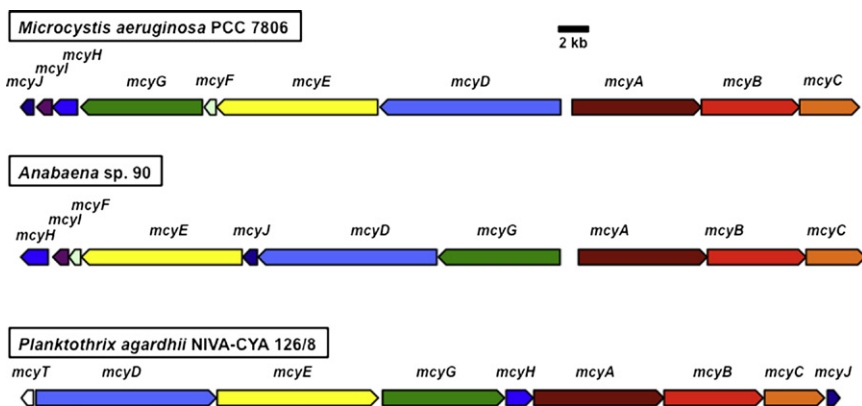
Microcystins are nonribosomal cyclic heptapeptides containing an unusual  $\beta$ -amino acid (Adda: (2*S*, 3*S*, 8*S*, 9*S*)-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid) and several nonproteogenic amino acids including *N*-methyldehydroalanine (Fig. 6.1). These toxins are produced by freshwater cyanobacteria belonging to different genera such as *Microcystis*, *Planktothrix*, or *Anabaena* (Pearson, Mihali, Moffitt, Kellmann, & Neilan, 2010). More than 90 variants of microcystins have been identified, with



**Figure 6.1** Structure of microcystin-LR. The most frequent variations encountered are shown schematically according to their position. Abbreviations: Hty, Homotyrosine; Hph, homophenylalanine; Hil, homoisoleucine; Har, homoarginine; D-mAsp, D-erythro-3-methylaspartate; DesMeAdda, the Adda moiety lacking a methyl group at position 9; MeDha, *N*-methyldehydroalanine; Dha, dehydroalanine.

variable amino acids at the different positions and variable degree of methylation (Welker & von Dohren, 2006). Positions 2 and 4 show the highest variability, and the most common form is microcystin-LR (leucine in position 2 and arginine in position 4, Fig. 6.1). Microcystins are hepatotoxins and their primary targets are eukaryotic protein phosphatases of types 1 or 2A (Dawson, 1998; MacKintosh, Beattie, Klumpp, Cohen, & Codd, 1990). Interestingly, the dehydroalanyl residue (position 7) of microcystin reacts specifically with an active site cysteine residue of protein phosphatase 1 (MacKintosh et al., 1990; Maynes et al., 2006; Meissner, Dittmann, & Borner, 1996). The unusual structure of microcystins suggested that they were products of NRPSs, thus facilitating the identification of the biosynthetic genes. A 2-kb DNA sequence coding for an NRPS was first identified in the genome of the strain *Microcystis aeruginosa* PCC 7806, a microcystin producer (Meissner et al., 1996), and genetic inactivation of this gene confirmed that it coded for an NRPS involved in microcystin biosynthesis (Dittmann, Neilan, Erhard, von Dohren, & Borner, 1997). The complete cluster was then sequenced in two *M. aeruginosa* strains, and several gene-inactivation experiments confirmed the implication of this cluster in the biosynthesis of microcystin (Nishizawa, Asayama, Fujii, Harada, & Shirai, 1999; Nishizawa, Asayama, & Shirai, 2000; Tillett et al., 2000). The *mcy* cluster was later sequenced in *P. agardhii* NIVA-CYA 126/8 (Christiansen, Fastner, Erhard, Borner, & Dittmann, 2003), and *P. rubescens* NIVA-CYA 98 (Rounge, Rohrlack, Nederbragt, Kristensen, & Jakobsen, 2009), and *Anabaena* sp. 90 (Rouhiainen et al., 2004). The 55-kb *mcy* cluster of *M. aeruginosa* PCC 7806 comprises 10 genes (*mcyA–J*, Fig. 6.2) arranged in two divergently transcribed operons, consisting of *mcyA–C* and *mcyD–J*. However, other promoters were identified upstream of *mcyE*, *mcyF*, *mcyG*, *mcyH*, *mcyI* and *mcyJ* (Kaebernick, Dittmann, Borner, & Neilan, 2002; Tillett et al., 2000). In the two other *mcy* gene clusters so far sequenced (Fig. 6.2), the genes *mcyA*, *mcyB*, and *mcyC* are similarly arranged, while some differences are observed for the other *mcy* genes. This has been interpreted as the consequence of probable horizontal gene transfers, supported by the presence of transposase genes close to the *mcy* clusters in *M. aeruginosa* and *Anabaena* strains (Rantala et al., 2004). Recombination events as well as point mutations and insertions or deletions, within the *mcy* cluster, have also been proposed to explain the variations observed (Pearson, Moffitt, Ginn, & Neilan, 2008).

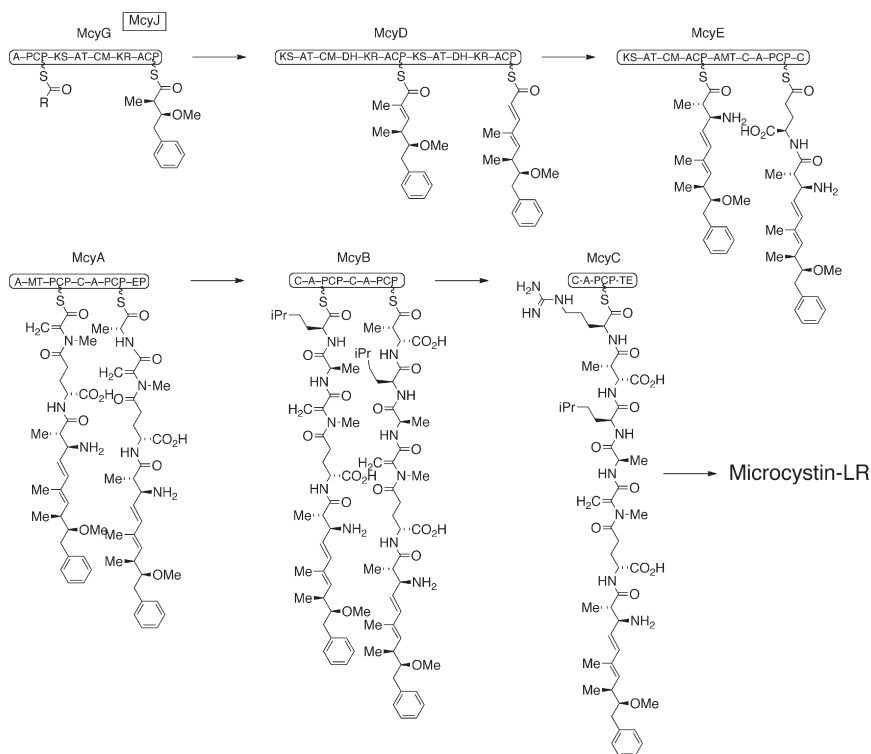
Not much is known concerning the regulation of the production of microcystins either at the transcriptional level or at the metabolic level.



**Figure 6.2** Comparison of three selected *mcy* gene clusters from different genera. Note that the *Planktothrix agardhii* *mcy* cluster lacks the *mcyI* and *mcyF* genes (implicated in the biosynthesis of erythro-2-methyl-D-Asp) and has a supplementary type-II thioesterase gene, *mcyT*. See the colour plate.

However, the transcription of the *mcy* genes is repressed in the dark and expressed by light, and thus, the production of microcystins occurs essentially during the light period (Straub, Quillardet, Vergalli, de Marsac, & Humbert, 2011).

A biosynthetic scheme, for microcystins, was proposed based on the bioinformatic analysis of the *mcy* cluster, involving two PKSs, one hybrid PKS/NRPS and three NRPSs, one tailoring enzyme and one ABC transporter (Tillett et al., 2000). The biosynthesis probably starts with the loading of phenyllactate on McyG followed by extension and methylation (Fig. 6.3). In vitro experiments conducted on the isolated adenylation domain and peptidyl carrier protein domain (A-PCP domains) of McyG showed that the preferred starter for McyG is probably phenyllactate rather than phenylacetate as first proposed (Hicks, Moffitt, Beer, Moore, & Kelleher, 2006). However, the carboxylate of the phenyllactate starter should be lost to give the Adda moiety by an unspecified step. McyJ, a tailoring enzyme, is believed to catalyse the O-methylation on the McyG product as evidenced by genetic inactivation of *mcyJ* (Christiansen et al., 2003). The chain is then loaded on McyD for extension on two modules, and then on McyE for extension, amino transfer and condensation of D-glutamate. The D-glutamate is tethered to the PCP by its  $\gamma$ -carboxylate, and thus, the next peptide bond is a  $\gamma$ -bond rather than an  $\alpha$ -bond. A first genetic study (Nishizawa, Asayama, & Shirai, 2001) attributed the glutamate racemase activity to McyF but



**Figure 6.3** The proposed biosynthesis for microcystin-LR. The actual starter of the PKS McyG (R on the figure) is not known. The domain abbreviations are defined in the abbreviations list.

in vitro experiments showed that this racemase uses aspartate rather than glutamate (Sielaff et al., 2003). Thus, the glutamate racemase gene is not part of the *mcy* cluster. The following steps are catalysed by three NRPSs, McyA, B, and C. They sequentially add five amino acids, *N*-methyldehydroalanine, *D*-alanine, *L*-leucine, *D*-erythro-3-methylaspartate with a  $\beta$ -peptide bond, and *L*-arginine. The origin of dehydroalanine is not known, but the *N*-methylation probably occurs on McyA as well as the epimerization of the next alanyl residue. The McyI and McyF proteins are thought to be involved in the biosynthesis of *D*-erythro-3-methylaspartate. The function of McyI was studied in vitro and it was shown that it is an NADPH-dependent dehydrogenase likely involved in the formation of *L*-threo-3-methylaspartate (Pearson, Barrow, & Neilan, 2007). It has been shown, in vitro, that McyF catalyses the racemization of aspartate, but it is not known if this enzyme epimerizes *L*-threo-3-methylaspartate to give *D*-erythro-3-methylaspartate.

Interestingly, *P. agardhii* NIVA-CYA 126/8 lacks the *mcyF* and *mcyI* genes, and it produces a microcystin variant containing D-aspartate in position 3. It is unclear how this cyanobacterium produces D-aspartate. The last enzyme, McyC catalyses the condensation of L-arginine and the final cyclization step, performed by the thioesterase (TE) domain. The *mcyH* gene probably codes for an ABC exporter and its inactivation caused an important decrease in the production of microcystin (Pearson, Hisbergues, Borner, Dittmann, & Neilan, 2004). In *P. agardhii* NIVA-CYA 126/8, another gene *mcyT* codes for a type-II TE likely involved in proofreading functions by hydrolysing misprimed PCP and acyl carrier protein (ACP) domains in the microcystin synthase (Christiansen, Molitor, Philmus, & Kurmayer, 2008).

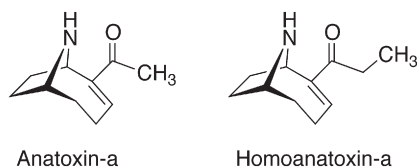
As noted above, more than 90 variants of microcystins have been identified. Axenic strains containing one *mcy* cluster produce two to four variants that differ in positions 2 (Leu or Arg), 3 (*erythro*-methyl-D-Asp or D-Asp) and 7 (*N*-methyl-Dha or Dha). Thus, the adenylation (A) domains of McyB must show some relaxed specificity and the methylation domain of McyA must be sometimes inactive. Within species and genera, the origin of the variants probably comes from recombination events in the *mcy* genes and, in particular, in the sequence coding for the A domains (Kaasalainen et al., 2012; Tooming-Klunderud, Fewer, et al., 2008; Tooming-Klunderud, Mikalsen, et al., 2008). These mutations will influence the amino acid activated at each position, leading to microcystin variants.

Nodularins are nonribosomal cyclic pentapeptides closely related to microcystins in terms of structure and biosynthesis. They are produced by cyanobacteria of the genus *Nodularia*, and, like microcystins, are hepatotoxic. They contain the Adda residue, like microcystins, and several variants have been identified, with various degree of methylation. The biosynthetic gene cluster, *nda*, has been identified in *Nodularia spumigena* and it shares strong similarities with the *mcy* gene cluster (Moffitt & Neilan, 2004). It has been suggested that the *nda* genes are recently derived from the *mcy* genes (Rantala et al., 2004).

#### **4.1.2. The neurotoxins anatoxin-a, homoanatoxin-a, and saxitoxins**

Anatoxin-a and homoanatoxin-a (Fig. 6.4) are two potent neurotoxins produced by freshwater cyanobacteria of different genera such as *Anabaena*, *Aphanizomenon*, *Oscillatoria*, *Phormidium*, *Cylindrospermum*, or *Raphidiopsis* (Osswald, Rellán, Gago, & Vasconcelos, 2007). These alkaloids act as potent agonist of the muscular nicotinic acetylcholine receptor provoking muscle paralysis and respiratory failure (Wonnacott & Gallagher, 2006).

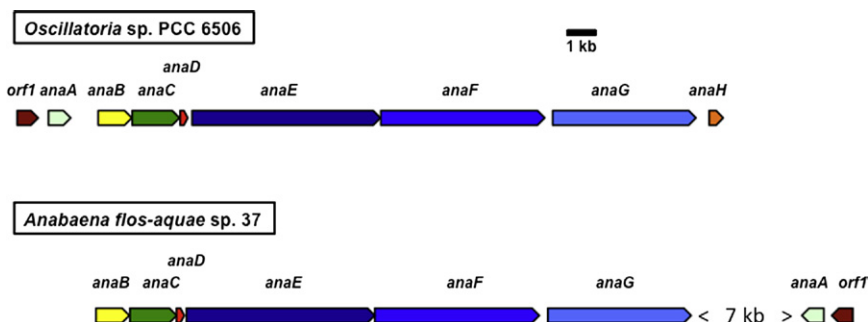




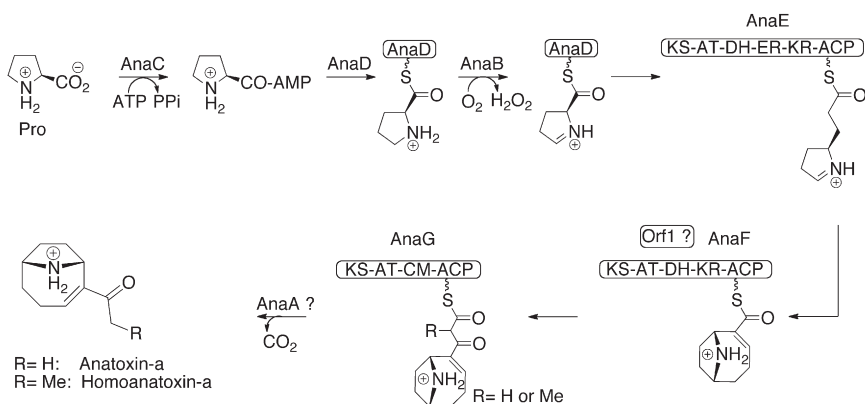
**Figure 6.4** The structure of anatoxin-a and homoanatoxin-a.

Deaths of animals due to ingestion of these toxins are regularly reported in the world (Cadel-Six et al., 2007). Although anatoxin-a was isolated in 1972, its biosynthesis has only been deciphered recently. In 2009, Méjean et al. used degenerate primers to amplify, by PCR, sequences coding for ketosynthase (KS) domain of PKSs, in the genome of *Oscillatoria* sp. PCC 6506, a homoanatoxin-a producer (Cadel-Six et al., 2009). They identified a 1.7-kb sequence, called *ks2*, that was specific for *Oscillatoria* strains producing anatoxin-a or homoanatoxin-a. This sequence was then found within a cluster of nine genes, in the genome sequence of *Oscillatoria* sp. PCC 6506 (Mejean et al., 2010). The cluster, designated *ana*, was proposed to be responsible for the biosynthesis of anatoxin-a and homoanatoxin-a, and a complete biosynthetic scheme for these alkaloids was thus proposed based on a combination of bioinformatic analysis, feeding experiments and in vitro biochemical experiments on isolated enzymes (Méjean et al., 2009). The *ana* cluster was afterwards identified in the genome of *Anabaena flos-aquae* sp. 37, an anatoxin-a producer (Rantala-Ylinen et al., 2011). The clusters are quite similar although two genes, *orf1* and *anaA*, are differently located in the clusters (Fig. 6.5). The genes *anaB*, *C*, *D*, *E*, *F* and *G* and the encoded proteins share strong sequence identities suggesting a common origin. Unfortunately, neither genetic inactivation of these *ana* genes nor transcriptional studies have yet been reported. No transporter has been identified for anatoxin-a, and it is thus assumed that the toxin is released when the cells lyse during senescence.

The biosynthesis of anatoxin-a and homoanatoxin-a starts from proline (Fig. 6.6), which is loaded on an ACP, AnaD and then oxidized to pyrroline-5-carboxyl-ACP, by AnaB. The biosynthesis then takes place on three consecutive PKSs, AnaE, F, and G. The first PKS, AnaE, should add one acetate unit that is completely reduced. The second PKS, AnaF, should add one acetate unit and should catalyse a Mannich cyclization that forms the bicyclic structure of anatoxin-a and homoanatoxin-a. It has been proposed, on the basis of bioinformatic analysis, that the protein coded by *orf1* participates in this cyclization reaction. The last PKS, AnaG should add the final



**Figure 6.5** The two *ana* gene clusters so far identified. See the colour plate.



**Figure 6.6** The proposed biosynthesis of anatoxin-a and homoanatoxin-a, in *Oscillatoria* sp. PCC 6506. The cyclization on AnaF might be catalysed by a tailoring enzyme encoded by the *orf1* gene although this has not been proved. The last steps, the hydrolysis and decarboxylation, remain elusive, although AnaA might be involved. The domain abbreviations are defined in the abbreviations list.

acetate unit, to yield 11-carboxyanatoxin-a or 11-carboxyhomoanatoxin-a, tethered to the ACP domain as thioesters. The methylation responsible for the formation of homoanatoxin-a is likely catalysed by a specific domain on AnaG. The last step would be the hydrolysis of the thioesters to give 11-carboxyanatoxin-a or 11-carboxyhomoanatoxin-a that would give the corresponding toxin by decarboxylation. It is not yet clear if AnaA, a type-II TE, is implicated in these steps, but 11-carboxyanatoxin-a has been actually detected in the extract of *Aphanizomenon issatchenkoi*, an anatoxin-a producer (Selwood, Holland, Wood, Smith, & McNabb, 2007). The initial steps of this biosynthesis, catalysed by AnaC and AnaB, have been completely reconstituted in vitro, thus validating the predicted functions (Mann, Lombard,

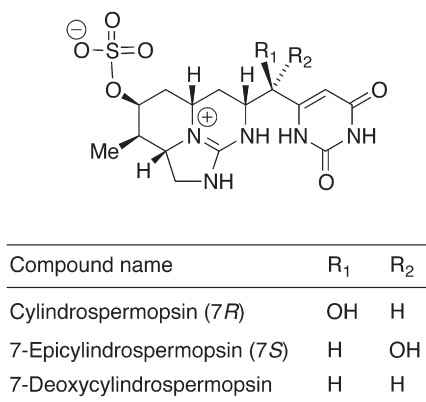
Loew, Mejean, & Ploux, 2011; Méjean et al., 2010). AnaC is an adenylation protein very specific for proline and AnaB is a flavoprotein oxidase homologous to isovaleryl-CoA dehydrogenase in terms of reaction mechanism and three-dimensional structure.

Saxitoxins and paralytic shellfish toxins are structurally closely related alkaloids that provoke the paralytic shellfish poisoning. There are more than 30 analogues of saxitoxin that have been identified (Kellmann et al., 2008; Wiese, D'Agostino, Mihali, Moffitt, & Neilan, 2010). These toxins act as blockers of the voltage-gated sodium and calcium channels. The biosynthesis of saxitoxin has been studied by feeding experiments and the putative biosynthetic genes have been identified in several cyanobacterial producers belonging to different genera: *Anabaena*, *Aphanizomenon*, *Cylindrospermopsis*, *Raphidiopsis*, and *Lyngbya* (Kellmann et al., 2008; Mihali, Kellmann, & Neilan, 2009; Murray, Mihali, & Neilan, 2011; Stucken et al., 2010). The strategy relied on using degenerate PCR amplifications targeting carbamoyltransferase genes because these enzymes were putatively involved in the biosynthesis (Kellmann et al., 2008). The cluster of genes spans 35 kb and there are 31 coding sequences. A biosynthetic pathway has been proposed on the basis of bioinformatic analysis, although none of the steps has been studied in vitro. Several studies have shown that the *sxt* clusters, from different species, have been remodelled after loss, rearrangements and recombination events (Murray et al., 2011).

#### 4.1.3. The cytotoxin cylindrospermopsin and its analogues

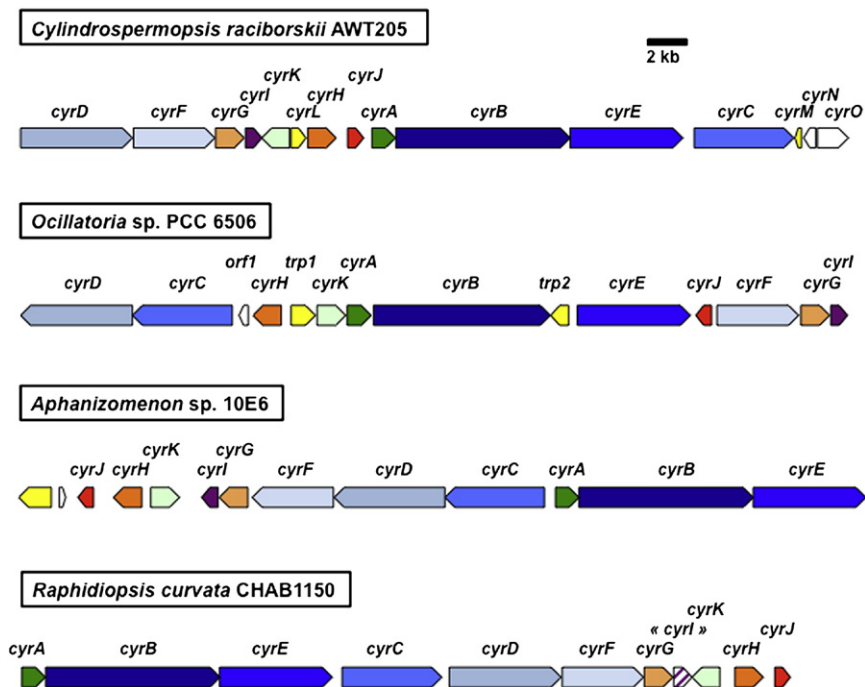
Cylindrospermopsin (Fig. 6.7) is an alkaloid that has been isolated from a fresh water cyanobacterium, *Cylindrospermopsis raciborskii*, that was isolated in a lake after a serious intoxication episode in Australia (Griffiths & Saker, 2003). Its chemical structure was revised when the two epimers at position 7 were synthesized (Heintzelman, Fang, Keen, Wallace, & Weinreb, 2001, 2002). In fact, three variants have been detected in cyanobacteria: 7-deoxycylindrospermopsin, cylindrospermopsin and 7-epicylindrospermopsin, depending on the substituents on carbon 7 (Fig. 6.7). Cylindrospermopsin and its natural analogues are cytotoxins, and provoke hepatotoxicity in humans (Froscio, Fanok, & Humpage, 2009). Several genera of cyanobacteria produce cylindrospermopsin or its analogues: *Anabaena*, *Aphanizomenon*, *Cylindrospermopsis*, *Oscillatoria*, *Raphidiopsis*, and *Umezakia* (Pearson et al., 2010).

The biosynthesis of cylindrospermopsin was first studied by feeding experiments and it was concluded that PKSs were involved and that the



**Figure 6.7** The structure of cylindrospermopsin and its natural analogues.

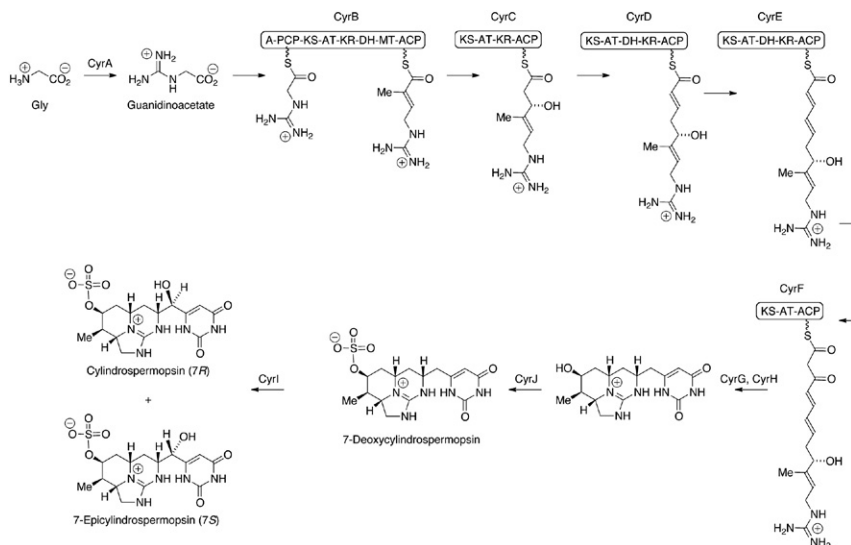
starter of these PKSs was guanidinoacetate (Burgoyne, Hemscheidt, Moore, & Runnegar, 2000). Using degenerate primers to amplify KS domains of PKSs, Kaplan et al. identified an 11-kb fragment containing three adjacent genes, including the *aoaA* (equivalent to *cyrA*) gene coding for the putative amidinotransferase, in *Aphanizomenon ovalisporum*, a cylindrospermopsin producer (Shalev-Alon, Sukenik, Livnah, Schwarz, & Kaplan, 2002). Later on, using gene walking technology, Neilan et al. sequenced the entire *cyr* cluster from *C. raciborskii* AWT 205 (Mihali, Kellmann, Muenchhoff, Barrow, & Neilan, 2008). The *cyr* cluster was later identified and sequenced in other cylindrospermopsin producers: *Aphanizomenon* sp. strain 10E6 (Stuken & Jakobsen, 2010), *Oscillatoria* sp. PCC 6506 (Mazmouz et al., 2010), and *Raphidiopsis curvata* CHAB1150 (Jiang et al., 2012). The four *cyr* clusters share strong identities but the genes are arranged differently (Fig. 6.8). Two genes, *cyrN* and *cyrO*, from *C. raciborskii* were not found in the other clusters and are thus likely not directly involved in the biosynthesis. *cyrN* codes for an adenylylsulfate kinase and this gene has been found in *Oscillatoria* sp. PCC 6506 genome but not in the *cyr* cluster. This activity is implicated in the formation of a universal sulphate donor (3'-phosphoadenylyl sulphate) and is likely not restricted to the biosynthesis of cylindrospermopsin. The *cyrO* gene was putatively annotated as a regulator gene but its function has not been studied. While no genetic inactivation experiments were reported to prove the function of the *cyr* genes, the function of the proteins CyrA and CyrI was demonstrated in vitro (see below in this section). However, a recent study described a natural inactivation of *cyrI* by insertion in *R. curvata*. This strain only produces 7-deoxycylindrospermopsin, validating the function of CyrI.



**Figure 6.8** The four *cyr* clusters so far identified. The transposase genes are coloured in yellow. The *cyrN* and *cyrM* genes in *Cyindrospermopsis raciborskii* were not found in the other *cyr* clusters. In *Raphidiopsis curvata*, *cyrI* is a pseudogene, and this strain produces 7-deoxycylindrospermopsin. See the colour plate.

The regulation of the production of cylindrospermopsin at the transcriptional level as well at the metabolic level has been studied by Kaplan and co-workers (Shalev-Malul et al., 2008). They identified an Abr-B-like protein that specifically binds to the promoter region between *cyrA* and *cyrC* genes. They also reported that light and nitrogen availability affected the transcription of these genes as well as the cylindrospermopsin production.

The biosynthesis of cylindrospermopsin has been proposed on the basis of feeding experiments and bioinformatics analysis of the biosynthetic genes (Fig. 6.9). It starts with the formation of guanidinoacetate catalysed by the amidinotransferase, *CyrA*, that has been studied in vitro as an isolated enzyme (Muenchhoff, Siddiqui, & Neilan, 2012; Muenchhoff et al., 2010). Then, guanidinoacetate is thought to be loaded on the PKS *CyrB* for extension. Four further PKSs (*CyrB*, *C*, *D* and *E*) are thought to extend the chain that will finally lead to the desulfated 7-deoxycylindrospermopsin.



**Figure 6.9** The proposed biosynthesis for cylindrospermopsin. The PKS intermediates are shown here as linear molecules. However, it is possible that some cyclization steps occur on the PKSs. The first step and the last step have been characterized in vitro. The protein CyrI shows variable stereospecificities from strain to strain. The domain abbreviations are defined in the abbreviations list.

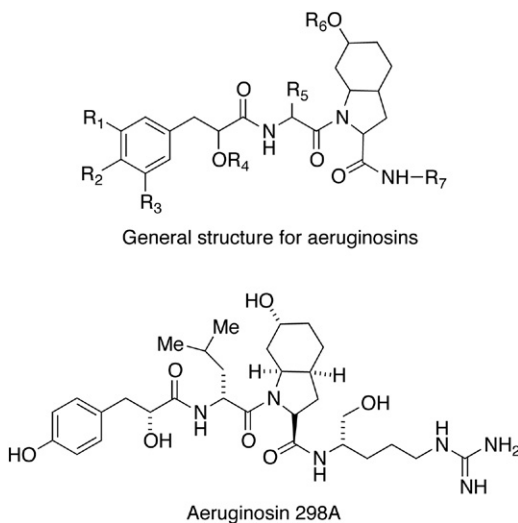
There are still uncertainties regarding the order of the PKSs steps because CyrD and CyrE share exactly the same domains and will thus catalyse the same reactions. Furthermore, the cyclization steps have been proposed to occur sequentially on the PKSs or after the PKS-mediated steps. There is no experimental data at this point to answer these interesting issues. CyrJ has been annotated as a sulfotransferase and is supposed to branch a sulphate group on the hydroxyl at position 12. Then, CyrI, a 2-oxoglutarate-dependent iron oxygenase, hydroxylates 7-deoxycylindrospermopsin to give cylindrospermopsin and 7-epicylindrospermopsin. The stereoselectivity of the isolated enzyme has been studied and varies from strain to strain (Mazmouz, Chapuis-Hugon, Pichon, Méjean, & Ploux, 2011).

## 4.2. Other Secondary Metabolites from Freshwater Cyanobacteria

### 4.2.1. Aeruginosins and aeruginosides

The aeruginosin family comprises diverse linear PK/NRPs produced by several *Microcystis* and *Planktothrix* strains. These peptides are inhibitors of serine and cysteine proteases with various potencies and selectivities (Ersmark,

DelValle, & Hanessian, 2008). All aeruginosins bear a common motif, the 2-carboxy-6-hydroxyoctahydroindole (Choi) residue, with variable residues at the N- and C-terminal ends, and diverse substituents on the phenyl ring, and on the Choi residue (Fig. 6.10). The biosynthetic genes for aeruginosins were identified by amplifying NRPS genes using a degenerate PCR approach, in *P. agardhii* (Ishida et al., 2007). The function of the identified genes was then confirmed by insertional genetic inactivation. The entire gene cluster, *aer*, was then obtained and sequenced in *P. agardhii* NIVA-CYA 126–8 (Ishida et al., 2007) and subsequently in diverse *Microcystis* and *Planktothrix* strains (Ishida et al., 2009; Rounge et al., 2009). The *aer* clusters contain the common *aer*ABCDEFGHI genes while other *aer* genes, *aer*JKLMN, have been identified in *Microcystis* strains only (Fig. 6.11). The *aer*CDEF are believed to be responsible for the production of the Choi amino acid from prephenate, and the *aer*ABGHIH genes are responsible for the biosynthesis of the linear peptide (Fig. 6.12). An interesting aspect in this biosynthesis is the presence of halogenases responsible for the halogenation of the phenyl ring of the primer, phenyllactate (Cadel-Six et al., 2008). The biosynthesis has been proposed on bioinformatic grounds and only one step, the AerD-catalysed step, has been actually studied in vitro (Mahlstedt, S., Fielding, E. N., Moore, B. S., & Walsh, C. T., 2010). There are, thus, many uncertainties and, in particular, the releasing step from the PKS AerG, which does not contain any TE domain.



**Figure 6.10** Generic structure for aeruginosins and the structure of aeruginosin 298A. The variable groups are:  $R_1$  and  $R_2$  = H, Cl;  $R_3$  = H, OH,  $\text{OSO}_3\text{H}$ ;  $R_4$  = H,  $\text{SO}_3\text{H}$ ,  $R_5$  = Leu, Ile, Phe, Tyr, Hty;  $R_6$  = H,  $\text{SO}_3\text{H}$ , xylose;  $R_7$  = diverse guanidines (see Ersmark, DelValle, & Hanessian, 2008).

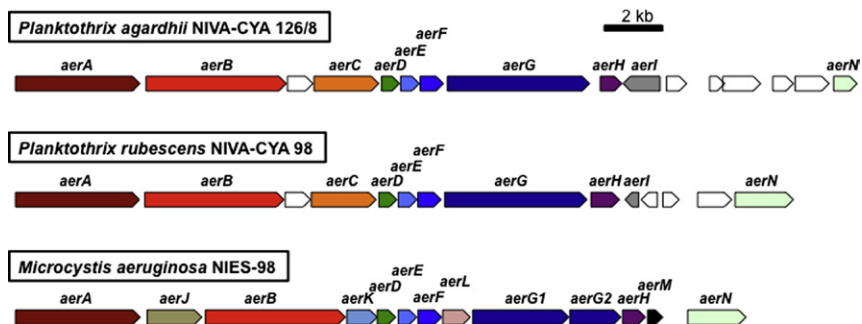


Figure 6.11 Selected *aer* gene clusters identified in different cyanobacteria. See the colour plate.

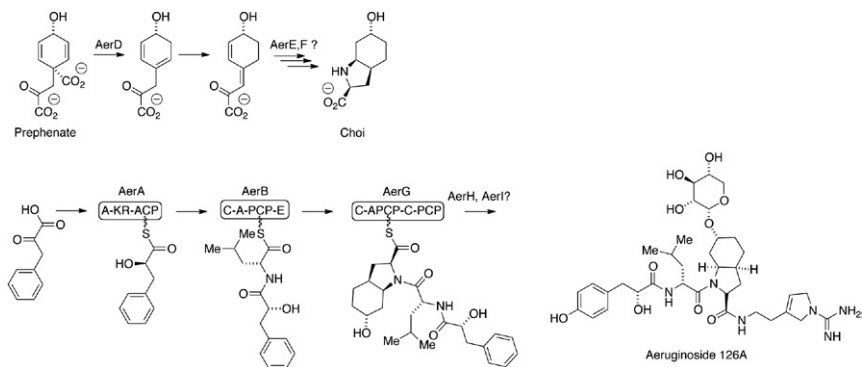


Figure 6.12 Proposed biosynthesis of the Choi residue and of aeruginoside 126A. The domain abbreviations are defined in the abbreviations list.

#### 4.2.2. Microginins

Microginins are linear PK/NRP isolated from *Microcystis* or *Planktothrix* species. They bear a  $\beta$ -amino acid in the N-terminal position, the 3-amino-2-hydroxy-decanoate (Ahda) (Welker & von Dohren, 2006). Several variants have been detected. These peptides are inhibitors of the angiotensin-converting enzyme. The cluster responsible for the biosynthesis of microginins in *P. rubescens* NIVA CYA-98 has been identified and called the *mic* cluster (Rounge et al., 2009). It consists of four genes, three coding for NRPS or PKS/NRPS hybrid, and one ABC transporter. Experimental evidences for the cluster function and the enzyme functions are lacking.

#### 4.2.3. Cyanopeptolins

Cyanopeptolins are cyclic nonribosomal depsipeptides containing a lactone bond. They are produced by cyanobacteria of different genera such as



*Microcystis*, *Anabaena*, or *Planktothrix*, and they contain an unusual 3-amino-6-hydroxy-2-piperidone unit (Ahp), which, in fact, corresponds to a cyclic glutamyl- $\gamma$ -semialdehyde residue (Welker & von Dohren, 2006). Several variants of cyanopeptolins have been detected, and they usually are protease inhibitors. Several clusters of genes responsible for cyanopeptolin biosynthesis have been identified in diverse strains, the *apd*, *mcn*, and *oci* clusters (see Table 6.1 and Rounge, Rohrlack, Tooming-Klunderud, Kristensen, & Jakobsen, 2007). These clusters code for NRPSs and show interesting variations within strains.

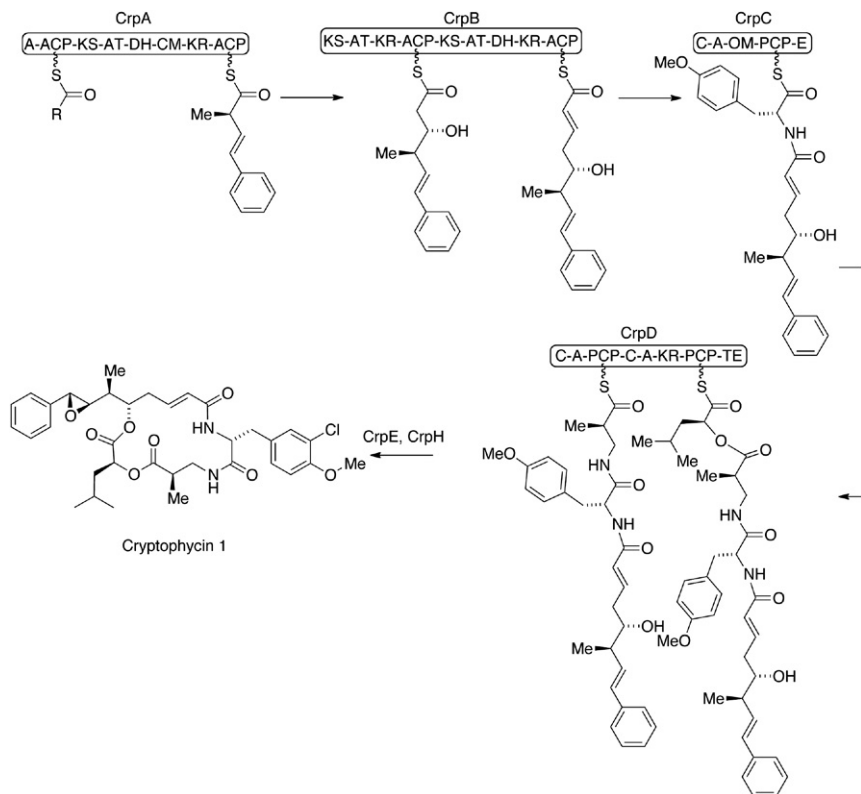
#### 4.2.4. Anabaenopeptins

Anabaenopeptins are cyclic hexapeptides with an ureido linkage between the variable residue at the N-terminal position and the D-lysine residue usually found in the cyclic moiety (Welker & von Dohren, 2006). There are many anabaenopeptin variants described, and these peptides that show protease inhibition activities are produced by diverse cyanobacteria. Five gene clusters responsible for the biosynthesis of anabaenopeptins from different strains have been identified – the *apt*, *ana* and *apn* clusters (see Table 6.1). They code for NRPSs and two studies have scrutinized the specificities of the isolated adenylation domains. In one strain, *Anabaena* sp. 90, the *apt* cluster contains two genes, *aptA1* and *aptA2*, that code for alternative starter modules, thus giving rise to different peptide with alternate residue (Tyr or Arg/Lys) at the N-terminal position (Rouhiainen, Jokela, Fewer, Urmann, & Sivonen, 2010). The other study, in *P. agardhii* NIVA CYA-126/8, showed that, within the *apn* cluster-encoded NRPSs, the first adenylation domain is promiscuous and accepts two residues as substrates (Arg or Tyr), giving thus two anabaenopeptin variants (Christiansen, Philmus, Hemscheidt, & Kurmayer, 2011).

### 4.3. Secondary Metabolites from Earth Cyanobacteria

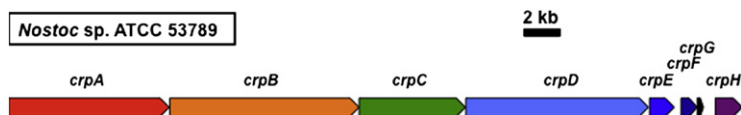
#### 4.3.1. Cryptophycins

Cryptophycins are depsipeptides produced by cyanobacteria of the genus *Nostoc* (Magarvey et al., 2006). More than 25 variants of cryptophycins have been identified, with cryptophycin 1 as the major representative (Fig. 6.13). The modifications concern several positions like the carbon-carbon double bonds, the epoxide, the methyl groups, and the configuration at some positions. These metabolites and semisynthetic analogues are promising anti-cancer drugs acting as tubulin-depolymerizing



**Figure 6.13** The structure of cryptophycin 1 and the proposed biosynthesis. The actual starter of the PKS CrpA, indicated as R on the figure, is not known. The domain abbreviations are defined in the abbreviations list.

agents. The biosynthesis of cryptophycin 1 has been studied using feeding experiments that served to identify the biosynthetic gene cluster in *Nostoc* sp. ATCC 53789 (Magarvey et al., 2006). The *crp* cluster consists of eight genes with four genes coding for PKSs and NRPSs (Fig. 6.14). The biosynthesis probably starts with the PKS CrpA although, like for microcystin biosynthesis, there are some doubts concerning the identity of the real starter (Fig. 6.13). The chain is then elongated on the CrpB, C and D synthases and the last TE domain performs the macrocyclization. Then, tailoring enzymes, like the epoxidase and the halogenase, modify the molecule to give cryptophycin 1. Some steps have been reconstituted in vitro and the isolated enzymes have been used to prepare cryptophycin in a chemoenzymatic way (Ding, Seufert, Beck, & Sherman, 2008, 2011).



**Figure 6.14** The gene cluster *crp* responsible for the biosynthesis of cryptophycin 1 in *Nostoc punctiforme* ATCC 53789. See the colour plate.

#### 4.3.2. Nostophycins

Nostophycin has been isolated from *Nostoc* sp. strain 152. It is a cyclic heptapeptide containing a  $\beta$ -amino acid residue, the (2*S*, 3*R*, 5*R*)-amino-2,5-dihydroxy-8-phenyloctanoate (Ahoa) (Fujii, Sivonen, Kashiwagi, Hirayama, & Harada, 1999). The biosynthetic cluster, *npn*, has been identified in the producing strain, and it consists of three genes coding for one PKS, one PKS/NRPS hybrid and one NRPS (Fewer et al., 2011). A biosynthetic scheme has been proposed based on the analysis of the sequence of the cluster. The specificities of the isolated adenylation domains were studied in vitro and they showed promiscuous selectivity, suggesting that variants of nostophycin should be observed (Fewer et al., 2011). As in the case of microcystin and cryptophycin biosynthesis, the starter of the first PKS, NpnA, is not known and the exact mechanism leading to the probable loss of one carbon remains elusive.

#### 4.3.3. Nostocyclopeptides and nostopeptolides

Nostocyclopeptides are cycloheptapeptides produced by *Nostoc* sp. ATCC 53789 (Golakoti, Yoshida, Chaganty, & Moore, 2001). These peptides contain a 4-methylprolyl residue and an imine linkage. The 33-kb cluster, *ncp*, has been identified and sequenced from the producer, and it codes for two NRPSs and for five other genes, among which the *ncpE* gene involved in the biosynthesis of the 4-methylproline amino acid (Becker, Moore, & Moore, 2004). The formation of imine linkage is rather unique and involves a reductive domain found at the C-terminal end of NcpB, the last PKS of this biosynthesis. Nostopeptolide A is a hybrid PK/NRP produced by the cyanobacterium *Nostoc* sp. GSV224, and it contains a leucylacetate unit and, like nostocyclopeptides, a 4-methylprolyl residue (Golakoti, Yoshida, Chaganty, & Moore, 2000). The biosynthetic cluster *nos* was identified and sequenced in *Nostoc* sp. GSV224 (Hoffmann, Hevel, Moore, & Moore, 2003). The genes, *nosE* and *nosF*, responsible for the biosynthesis of the 4-methylproline are part of the cluster, and a biosynthetic scheme for 4-methylproline was proposed starting from leucine, and confirmed in vitro (Luesch et al., 2003).

## 4.4. Toxins and Other Secondary Metabolites from Marine Cyanobacteria

Marine filamentous cyanobacteria produce a variety of secondary metabolites with interesting chemical structures and biological activities. These microorganisms have been largely used as a source of new drugs with a special emphasis on anti-cancer drugs. However, if many new metabolites have been identified, their biosynthesis remains largely unknown. But, several research groups have largely contributed to the understanding at the genomic and biochemical level of the biosynthesis of some remarkable marine cyanobacterial metabolites (Jones et al., 2010; Moore, Corbett, Patterson, & Valeriote, 1996; Nunnery et al., 2010). They have shown that the biosynthetic pathways are very diverse including NRPS, PKS, and NRPS-PKS hybrids. Excellent reviews cover this fascinating field and we have thus summarized here some interesting cases. The reader is referred to recent reviews covering the biosynthesis of lyngbyatoxins A–C, hectochlorin and barbamide (Jones, Gu, Sorrels, Sherman, & Gerwick, 2009; Jones et al., 2010; Nunnery et al., 2010).

### 4.4.1. Apratoxins

Apratoxins A–G share a core cyclic lipopeptide structure that is in fact a PK/NRP hybrid. These secondary metabolites have been extracted from diverse strains of *Lyngbya bouillonii* that are marine cyanobacteria and they show interesting cytotoxic bioactivities. The biosynthetic gene cluster for apratoxin A has recently been identified by using an interesting combination of different approaches by Gerwick and co-workers (Grindberg et al., 2011). These authors used a single cell as a PCR template to amplify the whole genome of the producer. The genome was then sequenced and the cluster was then identified using diverse screenings. The 58-kb *apr* cluster contains 12 genes, among which eight are coding for PKS or NRPS or hybrids. A biosynthetic pathway was proposed based on the bioinformatics analysis of the cluster sequence.

### 4.4.2. Curacins

Curacin A is a mixed PK/NRP produced by *Lyngbya majuscula*, containing interesting chemical motif: a cyclopropane ring and a thiazole ring derived from cysteine. This metabolite is of considerable interest because it is an antiproliferative compound with antitubulinin activity. Thus, analogues of curacin A might be used as anti-cancer drugs. The biosynthesis of this metabolite has been elucidated by feeding experiments

that allowed a prediction of the enzymes necessary to produce the molecule. The putative *cur* cluster was identified using a cosmid library from *L. majuscula* that was screened by southern hybridization. Several clusters were then identified, sequenced, and analysed by bioinformatics, and one cluster matched the predicted *cur* cluster. This 64-kb cluster contains 14 genes including several PKSs (Chang et al., 2004; Gu, Wang, Kulkarni, Geders, et al., 2009; Gu, Wang, Kulkarni, Gehret, et al., 2009). The last step of this biosynthesis is quite remarkable, and involves a sulfatation, followed by a decarboxylation and elimination, to give the terminal double bond. These steps have been studied in vitro using isolated domains (Gu, Wang, Kulkarni, Geders, et al., 2009; Gu, Wang, Kulkarni, Gehret, et al., 2009).

#### 4.4.3. *Jamaicamides*

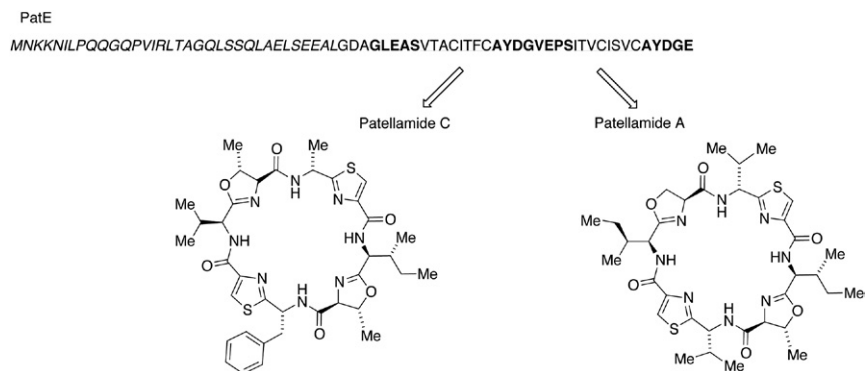
Jamaicamide A–C are mixed PK/NRPs produced by *L. majuscula*. They are blockers of the sodium channel and show toxicity towards fishes. The biosynthesis of these lipopeptides was studied by stable isotope incorporation, and the biosynthetic gene cluster was then identified by using a combination of PCR amplifications and southern hybridizations. The *jam* cluster contains 17 genes including eight genes coding for PKSs. A biosynthetic scheme was proposed based on the analysis of the *jam* cluster (Edwards et al., 2004). There are several interesting chemical motifs in jamaicamide, the triple carbon–carbon bond and the chlorovinyl group. The formation of the vinyl chloride motif has been studied and it parallels the formation of the cyclopropane ring of curacin (Gu, Wang, Kulkarni, Geders, et al., 2009; Gu, Wang, Kulkarni, Gehret, et al., 2009).

### 4.5. Ribosomal Peptides

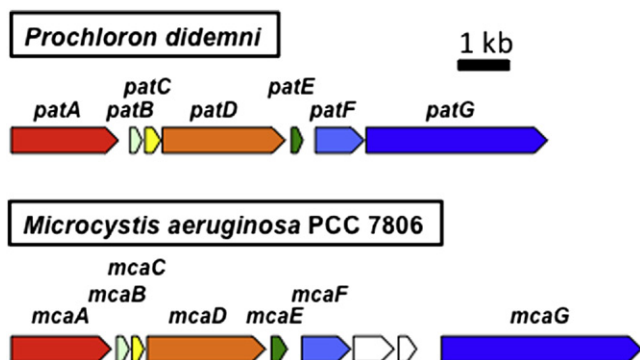
Cyanobacteria produce many RPs that are sometimes greatly modified by processing enzymes acting post-translationally. These RPs fall into several classes, such as the cyanobactins (Sivonen, Leikoski, Fewer, & Jokela, 2010), the microviridins (Ziemert, Ishida, Liaimer, et al., 2008; Ziemert, Ishida, Quillardet, et al., 2008), the bacteriocins (Wang, Fewer, et al., 2011; Wang, Xu, et al., 2011), or the lantipeptides (Li et al., 2010). Because the peptide core of the RP is derived from a precursor, the clusters of genes responsible of the biosynthesis of RP are usually small with only a few genes coding for the modifying enzymes. We shall briefly present two classes of RP, the cyanobactins and the microviridins with emphasis on the former.

### 4.5.1. Cyanobactins

Cyanobactins, such as patellamide or microcyclamide, are small cyclic RPs produced by cyanobacteria of different genera. These peptides usually contain oxazoline or thiazoline motifs or their oxidized derivatives, oxazole or thiazole, although there are examples of cyanobactins that do not contain any of these heterocycles (Sivonen et al., 2010). Cyanobactins are also sometimes prenylated on amino acid side chains of serine, threonine or tyrosine. These peptides show very diverse biological activities such as inhibition of proteases, or cytotoxicity. The cluster of genes responsible for the biosynthesis of patellamide (Fig. 6.15) was first identified in *Prochloron didemni* (Schmidt et al., 2005), and later, homologous cyanobactin clusters have been found in many cyanobacterial genomes, by genome mining (Donia & Schmidt, 2011). Indeed, the biosynthetic genes are conserved, although with some differences from genus to genus and strain to strain (Fig. 6.15). The biosynthesis of these peptides is not fully understood but the main steps have been deciphered (McIntosh, Donia, Nair, & Schmidt, 2011; McIntosh, Donia, Schmidt et al., 2010; McIntosh, Robertson, et al., 2010; McIntosh & Schmidt, 2010). The precursor, PatE in the case of patellamide, bears an N-terminal conserved leader sequence, followed by two sequences that will give the patellamide A and C, flanked by conserved recognition sequence motifs for the protease (Fig. 6.16). The precursor is first processed by the enzyme, PatD, responsible for the heterocyclizations of serine, threonine and cysteine residues. Then, the protease PatA cleaves off the N-terminal part of the precursor after the GLEAS or GVEPS recognition sequences. Then the PatG protease cleaves off the C-terminal AYDGE



**Figure 6.15** The structure of patellamide A and C and of their common precursor. The leader sequence is italicized, the protease recognition sequences are in bold type, and the cyanobactin sequences are underlined.



**Figure 6.16** Comparison of the cyanobactin clusters responsible for the biosynthesis of patellamide and microcyclamide. See the colour plate.

recognition sequence and performs the macrocyclization. These catalytic events lead to the cyclic peptide that will eventually be prenylated by the PatF or homologous enzymes. The fact that the leader sequence and the protease recognition sequences are conserved in the precursors of cyanobactins has led to successful predictions of cyanobactin structures in diverse cyanobacteria. Another interesting aspect of this pathway is that it is possible to create cyclic peptides in a combinatorial manner by introducing variable and unnatural sequences within the proteases recognition sequences in the precursor (Tianero, Donia, Young, Schultz, & Schmidt, 2012).

#### 4.5.2. Microviridins

Microviridins are RPs produced by different genera of cyanobacteria, such as *Microcystis*, *Oscillatoria*, or *Planktothrix*. These peptides bear a unique tri-cyclic structure with two lactone bonds and a lactam bond. They show interesting biological properties such as inhibition of proteases. The biosynthetic gene clusters, *mdn* and *mvd*, have been identified and sequenced in several strains (see Table 6.1). The *mdn* cluster from *M. aeruginosa* NIES 298 contains five genes. On this basis, a biosynthesis for microviridin B has been proposed: the precursor MdnA that contains an N-terminal leader sequence followed by the microviridin sequence is first processed by two ATP grasp proteins, MdnC and MdnB, in a sequential manner to form the three cyclic moiety (Philmus, Christiansen, Yoshida, & Hemscheidt, 2008; Philmus, Guerrette, Hemscheidt et al., 2009; Ziemert, Ishida, Liaimer, et al., 2008; Ziemert, Ishida, Quillardet, et al., 2008, Ziemert, Ishida, Weiz, Hertweck, & Dittmann, 2010). The peptide is then presumably cleaved and acetylated by

MdnD to give the final metabolite. The last gene, *mdnE*, codes for an ABC transporter. Interestingly, several putative microviridin precursors have been identified by genome mining in diverse cyanobacteria (Weiz et al., 2011).

## 4.6. Sunscreens

Cyanobacteria, like many other microorganisms, produce secondary metabolites to protect themselves against UV radiations. In cyanobacteria, these metabolites, the so-called sunscreens, are of two types: scytonemin and mycosporines (Gao & Garcia-Pichel, 2011a, 2011b).

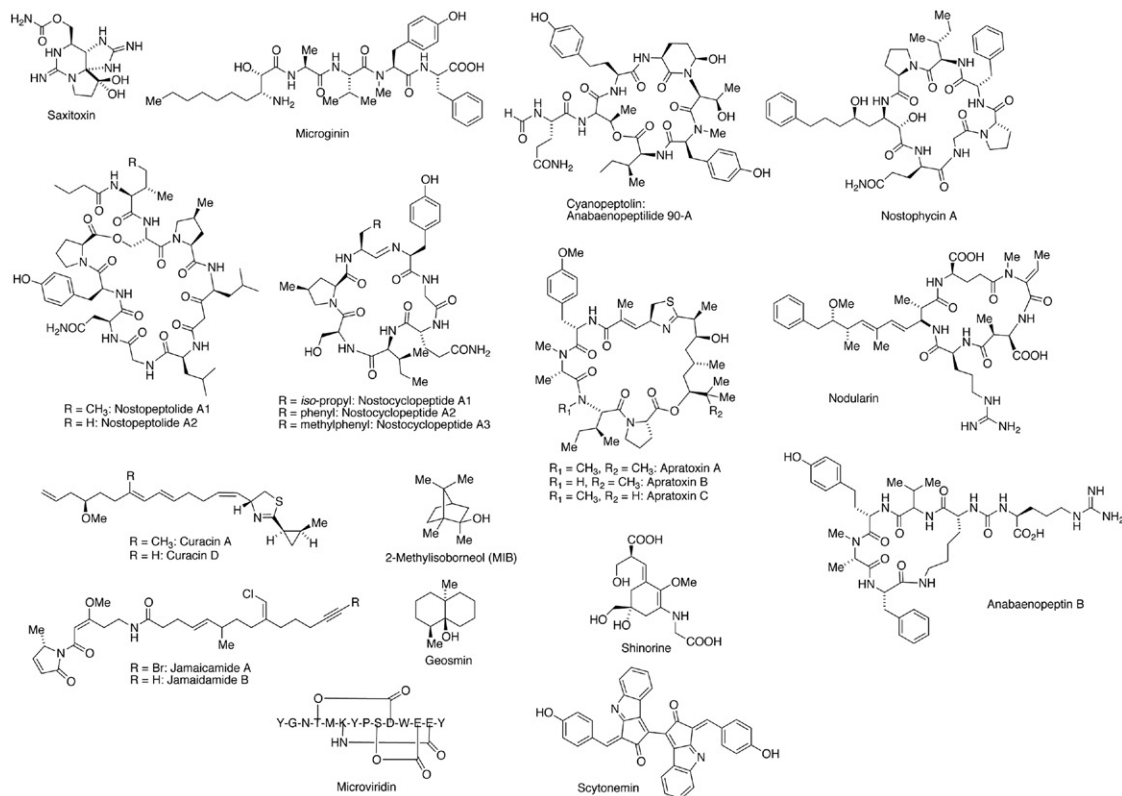
### 4.6.1. Scytonemin

The cluster of genes responsible for the production of scytonemin was identified in *Nostoc punctiforme* by random genetic inactivations (Soule, Stout, Swingley, Meeks, & Garcia-Pichel, 2007). The cluster comprises six genes, *scyA–F*, directly involved in the biosynthesis of scytonemin and several other genes likely involved in the formation of precursors. The biosynthesis of this metabolite was then studied in vitro using isolated enzymes, ScyA, B and C, and it was shown that scytonemin is produced from tryptophan and prephenate (Balskus & Walsh, 2008, 2009). The initial steps of the biosynthesis take place in the cytoplasm, whereas the final steps would occur in the periplasm. The final yellow pigment is excreted and deposited on the extracellular polysaccharide polymer. The *scy* cluster of genes has been detected in numerous cyanobacterial genera such as *Lyngbya*, *Anabaena*, and *Nodularia* (Soule et al., 2009).

### 4.6.2. Shinorine

Shinorine belongs to the mycosporine family of sunscreens and its biosynthesis has been studied in *Anabaena variabilis* ATCC 29413 and *N. punctiforme* ATCC 29133. A biosynthesis for shinorine, in *A. variabilis* ATCC 29413, has been proposed based on the bioinformatic analysis of the putative biosynthetic gene cluster, which comprises four genes. The entire gene cluster of 6.5 kb was expressed in *E. coli* and the transformant produced shinorine, thus validating the function of this cluster. The pathway was also supported by in vitro experiments, using isolated enzymes (Balskus & Walsh, 2010). The biosynthesis starts from sedoheptulose-7-phosphate, which is transformed into 4-deoxygadusol. This intermediate is in turn condensed with glycine to give mycosporine–glycine. Finally, an NRPS-like protein condenses mycosporine–glycine and serine to give shinorine. In *N. punctiforme* ATCC 29133, the last enzyme is lacking, and thus, the final metabolite





**Figure 6.17** Structure of the secondary metabolites presented in Table 6.1 that are not discussed in detail in the text.

is mycosporine–glycine (Gao & Garcia-Pichel, 2011a, 2011b). A comparative genomic study of this pathway in diverse cyanobacteria showed that the genes coding for the first three enzymes are conserved (Gao & Garcia-Pichel, 2011a, 2011b). However, a recent publication showed that there are probably, in *A. variabilis* ATCC 29413, two redundant biosynthetic pathways leading to 4-deoxygadusol, the precursor of shinorine (Spence, Dunlap, Shick, & Long, 2012).

## 4.7. Alkanes, Alkenes and Terpenes

### 4.7.1. Alkanes and alkenes

The biosynthesis of alkanes in cyanobacteria was elegantly deciphered by Schirmer, who used a genomic approach (Schirmer et al., 2010). By comparing the genome of cyanobacteria producing or not producing alkanes, potential biosynthetic genes were identified and finally, two candidate genes were found in *Synechococcus elongatus* PCC 7942. These two genes were found in other cyanobacteria, and they are clustered in the genome. They code for an acyl-ACP reductase and an aldehyde decarbonylase. The alkanes are thus produced from fatty acid by reduction to the aldehyde followed by decarbonylation, giving alkanes containing one less carbon than the parent fatty acid (the  $n - 1$  rule). Heterologous expression of these genes in *E. coli* confirmed their functions. The enzymology of the reduction/decarbonylation has also been studied in vitro, supporting the proposed functions (Krebs, Bollinger, & Booker, 2011). Interestingly, the biosynthesis of alkenes was recently identified in *Synechococcus* PCC 7002, a cyanobacterium that does not produce alkanes. In this cyanobacterium, a single gene of 8 kb codes for a PKS, which transforms C18 fatty acids into C19  $\alpha$ -olefins (Mendez-Perez, Begemann, & Pfleger, 2011). The function of this giant PKS was probed using genetic inactivation experiments.

### 4.7.2. Terpenes

Geosmin and 2-methylisoborneol (MIB) biosyntheses have been well studied because these compounds are odorant and their presence in water or foodstuff is sometimes problematic (Cane & Ikeda, 2012). Geosmin is formed from farnesyl-diphosphate by a single enzyme whose gene has been identified in *N. punctiforme* PCC 73102. The enzyme was cloned and expressed in *E. coli* and it produces geosmin together with other compounds, from farnesyl-diphosphate. MIB is produced in two steps from geranyl-diphosphate by a methyltransferase and a monoterpene cyclase. This biosynthesis has been extensively characterized in *Streptomyces* species.

Homologous genes coding for these two enzymes have been identified in *Pseudanabaena* and *Planktothricoides* strains, and they form an operon. However, neither inactivation nor in vitro studies were reported on this biosynthetic pathway, in cyanobacteria.



## 5. CONCLUSION

In the past decade, an impressive number of biosynthesis of cyanobacterial secondary metabolites has been unravelled. This is of course directly related to the number of genome sequences recently obtained for this group of bacteria. The biosynthetic pathways that have been elucidated are very diverse reflecting the chemical diversity of the secondary metabolites, and some very interesting enzymatic reactions have been discovered. In the near future, more cyanobacterial genomes will be released and it is expected that new clusters coding for biosynthetic enzymes involved in secondary metabolism will be discovered. However, there is a real need for more predictive bioinformatics to aid the annotation of these genes involved in secondary metabolism. In particular, it would be very useful to be able to predict the structure of the metabolite from the cluster sequence, although this might not be possible for complex metabolites. Better genetic tools for manipulating cyanobacteria will also be needed for studying the functions of the identified clusters of genes. Another interesting possibility is the heterologous expression of entire clusters to facilitate the chemical identification of the metabolite, but again this is not yet possible for large clusters of genes. The other main difficulty, in this field, resides in the isolation and structural identification of the metabolites, even using the up-to-date chemical tools. This is probably why there are still orphan clusters in cyanobacterial genomes, that is, clusters of genes coding for the biosynthesis of unknown secondary metabolites.

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# Assembly and Export of Extracellular Polymeric Substances (EPS) in Cyanobacteria: A Phylogenomic Approach

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## Abstract

Many cyanobacterial strains produce extracellular polymeric substances (EPS), mainly composed of polysaccharides that can remain associated to the cell or be released into the surrounding environment (released polysaccharides (RPS)). The particular characteristics of these EPS, such as the presence of two different uronic acids, sulphate groups

and high number of different monosaccharides (up to 13), make them very promising for biotechnological applications. Despite the increasing interest in these polymers, the information about their biosynthetic pathways is still limited. Studies performed in other bacteria revealed that the mechanisms of EPS assembly and export are relatively conserved, generally following the Wzy-dependent or the ABC-dependent pathways, which require the involvement of polysaccharide copolymerase (PCP) and outer membrane polysaccharide export (OPX) proteins. Our previous studies revealed that in cyanobacteria, the genes encoding these proteins occur in multiple copies, scattered throughout the genome, either isolated or in small clusters. However, it is necessary to identify other genes that may be related to this process, understand their genomic distribution, and reconstruct their evolutionary history. The data gathered here provide a first insight on the phylogenetic history of the EPS-related genes, and constitute a robust basis for subsequent studies aiming to optimize EPS production in cyanobacteria.



## 1. INTRODUCTION

Cyanobacteria possess an unusual prokaryotic envelope that combines the presence of an outer membrane, as in Gram-negative bacteria, with a thick and highly cross-linked peptidoglycan layer similar to that of Gram-positive organisms (Hoiczyk & Hansel, 2000; Stewart, Schluter, & Shaw, 2006). Many cyanobacterial strains also produce and export extracellular polymeric substances (EPS), mainly composed of polysaccharides, that can remain attached to the cell surface or be released into the surrounding environment (released polysaccharides (RPS)). The cyanobacterial EPS attached to the cell wall can be referred to as sheaths, capsules and slimes, according to their thickness, consistency and appearance (Pereira *et al.*, 2009). The sheath is usually a thin and dense layer that surrounds cells or cell groups and is visible in light microscopy without staining. The capsule generally consists of a thick and slimy layer intimately associated with the cell surface, characterized by its sharp outlines and for being structurally coherent to exclude particles (e.g. India ink). The slime refers to the mucilaginous material dispersed around the cells, usually not reflecting their shape (De Philippis & Vincenzini, 1998; Pereira *et al.*, 2009). Certain amounts of these external layers can be released into the surrounding medium, constituting the RPS. Differences observed in the monosaccharidic composition of sheaths and RPS of the same strain suggest that RPS are not merely due to the fragmentation of the external layers, but may also result from a divergent biosynthetic process (Li, Harding, & Liu, 2001; Micheletti *et al.*, 2008; Ortega-Calvo & Stal, 1994; Tease *et al.*, 1991). The production of the EPS boundary between the cell and its immediate environment requires a large amount of energy consequently, it is reasonable to assume that it

must confer some kind of selective advantage to the cells. Actually, several studies have shown that the presence of EPS can protect cyanobacteria against dehydration and UV radiation (Ehling-Schulz, Bilger, & Scherer, 1997; Ehling-Schulz & Scherer, 1999; Garcia-Pichel & Castenholz, 1991; Hill, Peat, & Potts, 1994; Potts, 1994, 1999, 2004; Shaw et al., 2003; Tamaru, Takani, Yoshida, & Sakamoto, 2005; Wright et al., 2005). In addition, it has been hypothesized that the EPS may play an important role in preventing the direct contact between the cells and toxic compounds, notably heavy metals and/or sequestering metal cations (or other nutrients) that are essential for cell growth but are present at low concentrations in the environment (Parker, Schram, Plude, & Moore, 1996; Sutherland, 1999). Overall, the role of these polymers seems to differ from strain to strain, being dependent on the physicochemical characteristics of the natural habitat or culture medium in which the organism grows.

Over the past decades, a considerable amount of efforts have been placed into the characterization of cyanobacterial EPS. The data gathered revealed that these polymers possess distinctive characteristics compared to those produced by other bacteria. Indeed, the EPS produced by cyanobacteria comprise a large number of different monosaccharides, with the majority of the polymers described possessing 6–13 different sugars. This feature contrasts with the polymers synthesized by other bacteria and macroalgae that usually contain around four (De Philippis & Vincenzini, 1998; Fischer, Schlosser, & Pohl, 1997; Pereira et al., 2009; Stengel, Connan, & Popper, 2011). Cyanobacterial EPS also frequently contain two different uronic acids (generally glucuronic and galacturonic) and sulphate groups, which are unusual in other bacterial EPS (Pereira et al., 2009; Sutherland, 1994), building a polymer particularly rich in negatively charged groups. In addition, many cyanobacterial polymers have a stronger hydrophobic behaviour, which is conferred by the presence of ester-linked acetyl groups (up to 12% of EPS dry weight), peptidic moieties and deoxysugars such as fucose and rhamnose (Neu, Dengler, Jann, & Poralla, 1992; Pereira et al., 2009; Shepherd, Rockey, Sutherland, & Roller, 1995). Altogether, these characteristics make cyanobacterial EPS very promising for biotechnological applications, such as the removal of heavy metals from polluted waters or as thickening, suspending or emulsifying agents (De Philippis, Colica, & Micheletti, 2011; Pereira et al., 2009). Despite the increasing interest on the cyanobacterial EPS and the growing awareness of their potential for biotechnological applications, the pathways leading to the production of these polymers remain scarcely known, limiting their manipulation and industrial

implementation. Therefore, it is crucial to elucidate the biosynthesis of the EPS in these organisms.



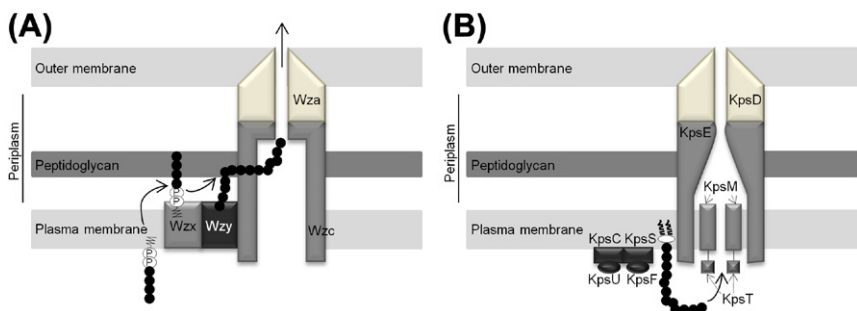
## 2. CONSERVED MECHANISMS OF BACTERIAL EPS PRODUCTION

In the past years, several studies have focused on the genetics of EPS biosynthesis in bacteria. However, cyanobacteria have not been thoroughly examined and, consequently, the information available is very limited. Studies performed with other bacteria point out that, regardless of the variety of surface polysaccharides produced, their biosynthetic pathways are relatively conserved, beginning with the activation of monosaccharides and its conversion into nucleotide sugars in the cytoplasm, and finishing with an assembled complex polymer outside the cell wall (Whitfield & Larue, 2008). This process requires the participation of three groups of proteins, including (1) enzymes involved in the biosynthesis of the nucleotide sugars, (2) glycosyltransferases, which catalyse the transfer of the nucleotide sugars from activated donor molecules to specific acceptors in the plasma membrane, and (3) proteins involved in EPS assembly and export (Reeves *et al.*, 1996). While the sugar activation/modification enzymes and the glycosyltransferases are strain specific, the proteins involved in the polymerization, assembly and export of the polymer seem to be well conserved, with most bacterial EPS being assembled and exported by one of the following pathways: (1) the synthase-dependent, (2) the Wzy-dependent, and (3) the ABC transporter-dependent (also referred to as Wzy-independent) (Cuthbertson, Kos, & Whitfield, 2010; Geremia & Rinaudo, 2005; Whitfield & Larue, 2008; Yother, 2011). Unquestionably, the Wzy-dependent pathway is the most widely distributed mechanism, being present in large number of bacteria (Whitfield, 2010; Whitfield & Larue, 2008). These pathways are not confined to the assembly and export of the EPS, but may also be involved in the formation of other surface polysaccharides, such as the O-antigen of LPS (Cuthbertson *et al.*, 2010; Mozzi, Savoy de Giori, & Font de Valdez, 2003). Regarding the synthase-dependent pathway, although it is used for the production of important biological polysaccharides, including bacterial cellulose, hyaluronan and alginate, the information available is still quite limited (Cuthbertson *et al.*, 2010; Franklin, Nivens, Weadge, & Howell, 2011; Raetz & Whitfield, 2002). It is known that the assembly and export of these polymers rely in the activity of a processive glycosyltransferase – the synthase – that serves as both a polymerase and an exporter. However,

the acceptor on which the polymer grows and the exact process by which the polymer is exported are yet to be identified (Cuthbertson et al., 2010). On the other hand, the Wzy- and ABC-dependent pathways involve the participation of a lipid acceptor upon which the polysaccharide is built (Cuthbertson et al., 2010; Geremia & Rinaudo, 2005). Both pathways are well-characterized and can be found in representative serotypes of *Escherichia coli*. As a consequence, the capsule assembly mechanisms of *E. coli* are regarded as paradigms for EPS assembly in a wide range of bacteria (Cuthbertson, Mainprize, Naismith, & Whitfield, 2009; Steenbergen & Vimr, 2008; Whitfield, 2006; Whitfield & Paiment, 2003; Whitfield & Roberts, 1999). These two processes are briefly described below.

## 2.1. Wzy-Dependent Pathway

The assembly and export of *E. coli* group 1 capsules constitutes the most well-studied example of the Wzy-dependent pathway (Fig. 7.1A). This process begins with the sequential transfer of the cytosolic nucleotide



**Figure 7.1** Schematic representation of the Wzy-dependent (A) and ABC-dependent (B) EPS assembly and export pathways. The Wzy-dependent pathway (A) begins with the assembly of oligosaccharide lipid-linked repeating units at the interface of the cytoplasm and the plasma membrane. Subsequently, the repeating units are translocated to the periplasmic side of the plasma membrane by the integral protein Wzx, and polymerized by Wzy. The polymerization reaction is influenced by the activity of the polysaccharide copolymerase (PCP) protein Wzc, which forms a complex with the polysaccharide export (OPX) protein Wza that allows the export of the polymer. In the ABC-dependent pathway (B), the polysaccharide is fully polymerized in the cytoplasmic face of the plasma membrane before being translocated through the plasma membrane by an ABC transporter comprising two transmembrane domains (KpsM) and two nucleotide-binding domains (KpsT). The participation of KpsC, KpsS, KpsF, and KpsU in this process is still unclear. The export of the polymer through the periplasm and outer membrane is performed by the PCP protein KpsE and the OPX protein KpsD (analogous to the Wzy-dependent pathway) (Cuthbertson et al., 2009, 2010; Whitfield, 2006). For colour version of this figure, the reader is referred to the online version of this book.



sugars into a lipid carrier located at the plasma membrane. The newly synthesized repeating units are then translocated to the periplasmic face of the plasma membrane by the integral protein Wzx, where they are polymerized by another integral membrane protein – Wzy. This reaction is influenced by the activity of the polysaccharide copolymerase (PCP) protein Wzc since it requires its interaction with the outer membrane polysaccharide export (OPX) protein Wza. These proteins form a complex that spans the cell envelope, thereby providing a molecular scaffold for the export of the polymer (Cuthbertson *et al.*, 2009; Whitfield, 2006).

## 2.2. ABC-Dependent Pathway

The assembly and export of *E. coli* group 2 capsules follow the ABC-dependent pathway (Fig. 7.1B). In this process, the polysaccharide is fully polymerized in the cytoplasmic face of the plasma membrane by the sequential addition of sugar residues to the nonreducing terminus of the nascent polymer. The complete molecule is then exported through the plasma membrane by an ABC transporter, comprising two transmembrane domains and two nucleotide-binding domains, which are encoded by *kpsM* and *kpsT* genes, respectively. Other proteins, such as KpsC, KpsS, KpsE, and KpsU, also participate in this process, but their precise role is still unclear. The export of the polymer through the periplasm and outer membrane requires the activity of the PCP protein, KpsE, and the OPX protein, KpsD, which may form a transenvelope assembly machine analogous to that of Wza and Wzc in the Wzy-dependent pathway (Cuthbertson *et al.*, 2010, 2009; Whitfield, 2006).

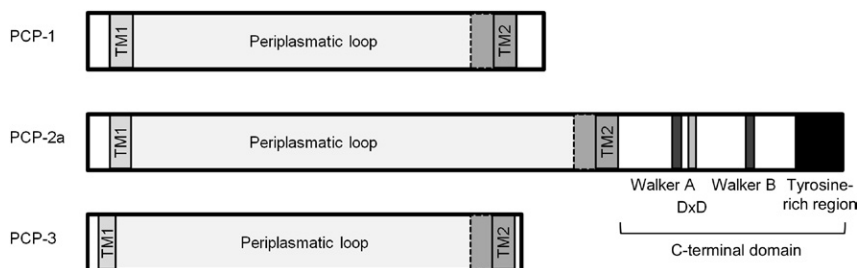
## 2.3. Role of PCP and OPX Proteins in EPS Assembly and Export

Despite the differences observed for the Wzy- and ABC-dependent assembly and export pathways, in the Gram-negative bacteria, both processes require the involvement of members of the PCP and OPX protein families for the translocation of the polymer to the cell surface. The structure, function, and phylogeny of the PCP and OPX proteins involved in this process were recently reviewed, providing new insights into its putative functions (Cuthbertson *et al.*, 2009).

### 2.3.1. Characteristics of the PCP proteins

The PCP proteins are essential for the assembly and export of several bacterial surface polysaccharides (Morona, Van Den Bosch, & Daniels, 2000).

The members of this protein family are characterized by the presence of two transmembrane regions (TM1 and TM2) flanking a large hydrophilic loop located in the periplasmic side of the plasma membrane (Cuthbertson et al., 2009; Morona et al., 2000). In addition, they also contain a proline- and glycine-rich domain preceding and overlapping the TM2 (Cuthbertson et al., 2009; Morona, Purins, Tocilj, Matte, & Cygler, 2009). The PCP proteins can be distinguished according to the type of polysaccharide, association with the Wzy or ABC-dependent assembly and export pathways, coiled-coil prediction profile and presence of characteristic domains (Fig. 7.2) (Morona et al., 2009, 2000). The PCP-1 family comprises the Wzz proteins, which participate in the biosynthesis of the O-antigen of LPS and the enterobacterial common antigen by the Wzy-dependent pathway (Morona et al., 2009, 2000; Whitfield, 2010). The PCP-2 refers to the proteins involved in the polymerization and export of high molecular polysaccharides, including the EPS, also following the Wzy-dependent pathway. The PCP-2 of Gram-negative bacteria, including Wzc, is designated as PCP-2a (Morona et al., 2000). The members of this group possess an additional carboxy-terminal cytoplasmic domain, which contains the Walker Box A and B motifs that are found in a variety of ATP- and GTP-hydrolysing proteins (Cuthbertson et al., 2010;



**Figure 7.2** General characteristics of the polysaccharide copolymerase (PCP) family proteins. The members of this family possess two transmembrane regions (TM1 and TM2) flanking a large periplasmic loop and a proline- and glycine-rich domain that precedes and overlaps the TM2 (depicted in grey). PCP-1 proteins participate in the biosynthesis of the O-antigen of LPS following the Wzy-dependent pathway. The PCP-2a are involved in EPS assembly and export in Gram-negative bacteria, also following the Wzy-dependent pathway. The members of this group possess an additional carboxy-terminal cytoplasmic domain, containing the Walker Box A and B motifs, a DXD signature, and a carboxy-terminal tyrosine rich region. PCP-3 proteins participate in the ABC-dependent EPS assembly and export systems. The PCP-1 and PCP-3 proteins are smaller than the PCP-2a, lacking the additional carboxy-terminal cytoplasmic region (Cuthbertson et al., 2009; Morona et al., 2000, 2009; Whitfield, 2010).

Soulat *et al.*, 2007; Walker, Saraste, Runswick, & Gay, 1982). Interestingly, in some of these organisms, the additional C-terminal domain is encoded by a separate gene, similar to what is observed for the PCP-2 proteins of Gram-positive bacteria, referred to as PCP-2b (Cuthbertson *et al.*, 2009; Morona *et al.*, 2000). Most PCP-2a and PCP-2b proteins also contain a tyrosine kinase domain located in the additional carboxy-terminal cytoplasmic region. In the case of Wzc, the tyrosine kinase domain is autophosphorylated at several C-terminal tyrosine residues, being subsequently dephosphorylated by the Wzb phosphatase (Wugeditsch *et al.*, 2001). Current working models propose that Wzc must cycle between its phosphorylated and nonphosphorylated forms to sustain the synthesis of the *E. coli* group 1 capsule polysaccharides. However, other PCP-2a proteins, such as the BcenP\_01003624 from *Burkholderia cenocepacia* lack the C-terminal tyrosine residues, and others, including the *Xanthomonas campestris* GumC, have no kinase domain at all, even though they participate in the EPS assembly and export. These observations raised the hypothesis that the PCP-2a kinase activity may, in fact, be related to earlier steps of the biosynthetic pathway. Despite these inferences, more information is needed to unveil the exact role of the kinase domain. The last group of PCP proteins, PCP-3 family, refers to the PCP representatives that participate in the ABC-dependent assembly and export systems, including the KpsE. These proteins are smaller than the PCP-2a, lacking the additional carboxy-terminal cytoplasmic region (Morona *et al.*, 2000).

### 2.3.2. Characteristics of the OPX proteins

The last steps of EPS assembly and export also require a member of the OPX protein family. These proteins are characterized by the presence of a polysaccharide export sequence (PES) domain (IPR003715; pfam 02563) (Cuthbertson *et al.*, 2009). The Wza protein of the Wzy-dependent pathway is an integral outer membrane lipoprotein, and structural studies revealed that it forms a ring-like octameric complex that provides the translocation channel across the outer membrane (Dong *et al.*, 2006; Drummelsmith & Whitfield, 2000). The periplasmatic domain of Wza specifically interacts with the periplasmatic domain of Wzc, leading to a conformational change in both proteins during the complex formation (Collins *et al.*, 2006, 2007; Ford *et al.*, 2009). The existing data suggest that in Wza, it is the PES domain that undergoes a conformational change (Collins *et al.*, 2007), allowing the translocation of the polymer

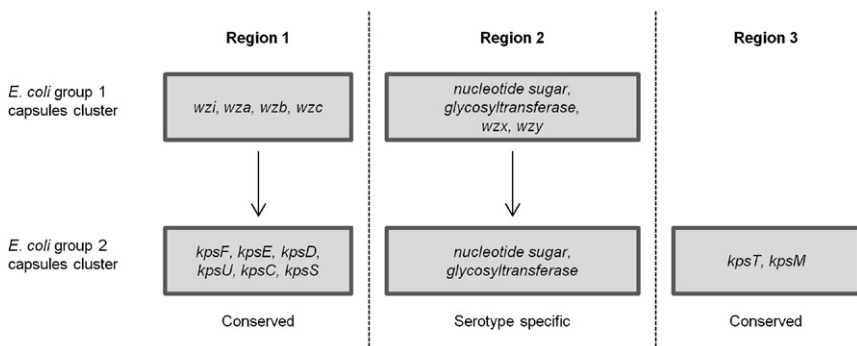
independently of its polysaccharidic structure. Likewise, the ABC-dependent assembly and export pathway requires the participation of the KpsD. Although this protein possesses the PES domain characteristic of the OPX proteins, it is not a lipoprotein and shares low sequence similarity with Wza (Cuthbertson et al., 2009). Considerably, less is known about a possible interaction between the OPX and PCP-3 components involved in the ABC-dependent pathway. However, existing data suggest that KpsD and KpsE may form a complex similar to that of Wza and Wzc (Cuthbertson et al., 2009).

## 2.4. Organization and Phylogeny of Gene Clusters Involved in EPS Biosynthesis

In most EPS-producing bacteria, the genes encoding the proteins involved in the synthesis, assembly and export of the EPS are located in the same genetic loci, exhibiting conserved features (Yother, 2011). In *E. coli*, the genetic loci encoding the proteins involved in the production of group 1 capsules, following the Wzy-dependent pathway, comprise two distinct regions. The 5' region contains conserved genes, including *wza*, *wzb*, and *wzc*. The 3' region is serotype specific and encodes the enzymes involved in the biosynthesis of the nucleotide sugars, glycosyltransferases and Wzx and Wzy proteins. While the genes related to nucleotide sugar metabolism and those encoding the glycosyltransferases are serotype specific, determining the monosaccharidic composition of the capsules produced, the *wzy* and *wzx* are always present, defining the pathway (Whitfield, 2006). This 3' region is identical in loci involved in the production of other polysaccharides by the Wzy-dependent pathway, such as the *E. coli* group 4 capsules and the O-antigen of LPS in different bacteria (Whitfield, 2006).

The organization of the genetic loci involved in the production of group 1 capsules can be found in other organisms, including several strains of the *Burkholderia* genus (Ferreira et al., 2010; Moreira et al., 2003) and *Klebsiella pneumoniae* (Rahn, Drummelsmith, & Whitfield, 1999). The gene clusters of *E. coli* and *Klebsiella* are highly conserved in organization and nucleotide sequence, raising the hypothesis of horizontal gene transfer (HGT) events between these organisms. The same organization can also be found in Gram-positive bacteria, such as *Streptococcus pneumoniae* (Yother, 2011) and several lactic acid bacteria (De Vuyst, De Vin, Vanningelgem, & Degeest, 2001; Jolly & Stinglele, 2001), despite the inherent differences related to the cell wall structure.

The conserved organization of group 1 capsules-related clusters appears to be reminiscent of the modular structure of the genetic loci required for the production of the ABC-dependent group 2 capsules in *E. coli* (Fig. 7.3), which comprise three distinct regions (Rahn *et al.*, 1999). Region 1 encodes the KpsF, E, D, U, C, and S, which are necessary for translocation of the polysaccharide, thus playing similar functions as those played by Wza, Wzb, and Wzc in the group 1 capsule-related clusters. The central region 2 is serotype specific and is responsible for the biosynthesis and polymerization of the polymer. This region is equivalent to the 3' region of group 1 capsule-related clusters, but lacks the *wzy* and *wzx*. Finally, region 3 comprises genes encoding components that are not required in the Wzy-dependent machinery, namely *kpsM* and *kpsT*, which encode the ABC transporter that characterizes the pathway. The gene products of the regions 1 and 3 are conserved, functioning independently of the structure of the capsule polysaccharide (Whitfield, 2006; Whitfield & Roberts, 1999). Similar organization is observed for the loci encoding the proteins



**Figure 7.3** Genetic organization of the loci encoding the proteins involved in the production of *E. coli* group 1 capsules (following the *wzy*-dependent pathway) and *E. coli* group 2 capsules (following the ABC-dependent pathway). In the first case, the 5' region of the loci usually contains the conserved genes *wzi* (coding a protein involved in surface attachment of the capsule), *wza*, *wzb*, and *wzc*, whereas the 3' region is serotype specific and encodes the enzymes involved in the biosynthesis of the nucleotide sugars, glycosyltransferases and the Wzx and Wzy proteins (Rahn *et al.*, 1999; Whitfield, 2006). The genetic loci required for the production of group 2 capsules comprises three regions. Region 1 encodes the KpsF, E, D, U, C and S (similar functions as those played by Wza, Wzb and Wzc). Region 2 is equivalent to the 3' region of group 1 capsule-related clusters, but lacks the *wzy* and *wzx*. Region 3 comprises the *kpsM* and *kpsT* encoding the ABC transporter that characterizes this pathway. The gene products of the regions 1 and 3 are conserved, functioning independently of the monosaccharidic composition/structure of the capsule polysaccharide (Rahn *et al.*, 1999; Whitfield, 2006; Whitfield & Roberts, 1999).

involved in synthesis and assembly of group 3 capsules, also by the ABC-dependent pathway, although fewer characteristic conserved genes are present and their positions and relative order differ from that observed for group 2 capsules-related loci (Whitfield, 2006).



### 3. GENETICS OF CYANOBACTERIAL EPS PRODUCTION: CURRENT KNOWLEDGE

The first insight into the genes encoding proteins involved in EPS assembly and export (hereafter referred to as EPS-related genes) in cyanobacteria was obtained recently (Pereira et al., 2009). In that study, the authors performed an *in silico* analysis of available cyanobacterial genome sequences, revealing the existence of genes coding for proteins that possess the conserved domains involved in bacterial EPS assembly and export. The results obtained also showed that in cyanobacteria, the EPS-related genes often occur in multiple copies scattered throughout the genomes, either isolated or in small clusters (Fig. 7.4) (Pereira et al., 2009).

Although gene redundancy is not unusual in cyanobacteria (Larsson, Nylander, & Bergman, 2011), the pattern observed for EPS-related genes is different from that of other bacteria, where the EPS-related genes are usually clustered (De Vuyst & Degeest, 1999; Ferreira et al., 2010; Jolly & Stingle, 2001; Rahn et al., 1999; Roberts, 1996; Whitfield, 2006). In general, as the complexity of the cyanobacterial strain/size of genome increases, more copies of the EPS-related genes are found (Pereira et al., 2009). It is possible that some of these genes encode proteins that play similar functions in closely related pathways, such as the production of O-antigen of the LPS, thus possessing the same functional domains and/or annotation. However, this hypothesis does not account for the multiple genes encoding putative OPX proteins since, up to this moment, there is no evidence of an interaction between the PCP-1 proteins involved in the assembly and export of the O-antigen with a Wza/KpsD homologue. Nevertheless, the presence of multiple genes coding for OPX proteins has also been reported for other bacteria, including *Burkholderia* and *Bacteroides* species (Cuthbertson et al., 2009). In the case of heterocystous strains, it is also necessary to take into consideration the biosynthesis of the polysaccharidic layer surrounding the heterocysts (Cardemil & Wolk, 1976, 1979, 1981a, 1981b; Dunn & Wolk, 1970). The identification of genes encoding proteins possessing the domains typically found in Wzx and Wzy, both characteristic of the Wzy-dependent pathway, strongly suggests that cyanobacterial EPS production follows this mechanism (Pereira et al., 2009;

Whitfield & Larue, 2008). Using this information, a working model for the assembly and export of cyanobacterial EPS following the general steps of the Wzy-dependent pathway was proposed (Pereira et al., 2009).

Despite these first insights on the mechanisms underlying EPS production in cyanobacteria, it is still necessary to understand, from a biological perspective, the existence of the multiple copies of EPS-related genes, as well as their organization and regulation within the cyanobacterial



genomes. To achieve that, it is essential to broaden this analysis to a larger group of cyanobacterial strains, which will provide a robust basis for the reconstruction of the phylogenetic history of cyanobacterial EPS-related genes, and consequently, allow the optimization of the EPS production mechanisms.

### 3.1. Phylogeny of Genes Encoding Proteins Involved in Cyanobacterial EPS Assembly and Export

The recent high-throughput sequencing techniques have generated large amounts of genetic data that can be used to reconstruct the evolutionary history of the EPS-related genes in cyanobacteria. To guarantee a thorough analysis, 24 cyanobacterial genome sequences (available at NCBI – National Centre for Biotechnology Information; database March 2012) were selected. The genomes belong to strains from different orders/subsections, displaying distinct morphologies, and that were isolated from various ecological niches.

To start our study, the conserved domains present on the proteins involved in well-characterized systems of bacterial EPS assembly and export, and that follow the Wzy- (Wz\_ homologues) or ABC-dependent (Kps\_ homologues) pathways (see above, sections 2.1. and 2.2.), were identified using the InterProScan Sequence Search available at the EMBL-EBI (European Bioinformatics Institute) (Hunter et al., 2012). Once the domains were identified, their presence or absence was investigated in the selected cyanobacterial strains, using the InterPro Protein Sequence Analysis & Classification Tool, also available at the EMBL-EBI. This analysis yield a large number of putative cyanobacterial Wz\_ and Kps\_ homologues (Tables 7.1 and 7.2).

Interestingly, proteins possessing the Wzy characteristic domain were found in all strains analysed, suggesting that cyanobacterial EPS assembly and export should, in most cases, proceed via this mechanism. This hypothesis is supported by the higher abundance of Wz\_ homologues in cyanobacteria, with several strains possessing all the Wz\_ surveyed. Despite that, nine strains, from unicellular to filamentous heterocystous, lack identifiable Wzx homologues, raising the hypothesis that the cyanobacterial EPS assembly and export systems may not fit exactly in the existing models. The postulation of a Wzy-dependent related pathway in cyanobacteria is reinforced by the low number of putative KpsC and KpsS identified, which could only be found in *Synechocystis* sp. PCC 6803, *Nostoc punctiforme* PCC 73102, and *Cylindrospermopsis raciborskii* CS-505. Although the exact functions of these proteins are yet undetermined, in *E. coli*, they were shown to be essential for



**Table 7.1** Putative cyanobacterial proteins involved in EPS assembly and export following the Wzy-dependent pathway

Putative protein		Wza	Wzb	Wzc	Wzx	Wzy
InterPro*		IPR003715	IPR000106 IPR017867 IPR023485	IPR003856 IPR005702 IPR005701	IPR002797	IPR007016
Chroococcales	<i>Cyanothece</i> sp. CCY 0110	CY0110_03539 CY0110_28374	CY0110_12312 CY0110_02244	CY0110_29394† CY0110_27525† CY0110_05934†	CY0110_07304	CY0110_26203 CY0110_11127
	<i>Cyanothece</i> sp. ATCC 51142	cce_4188 cce_1803	cce_1168 cee_2998	cce_1468	No hits	cce_4302 cce_1827
	<i>Cyanothece</i> sp. PCC 7822	Cyan7822_0713 Cyan7822_0909 Cyan7822_3789	Cyan7822_2153 Cyan7822_5306	Cyan7822_0712 Cyan7822_1903 Cyan7822_0908 Cyan7822_5718	Cyan7822_2783 Cyan7822_0910	Cyan7822_2928 Cyan7822_4837 Cyan7822_4891 Cyan7822_4978
	<i>Microcystis</i> <i>aeruginosa</i> NIES-843	MAE_37150 MAE_41410	MAE_00280 MAE_02540	MAE_32940 MAE_41520	MAE_32920 MAE_41540	MAE_24030 MAE_36740 MAE_41560
	<i>Prochlorococcus</i> <i>marinus</i> str. MIT 9301	No hits	P9301_17831	No hits	No hits	P9301_13971
	<i>Prochlorococcus</i> <i>marinus</i> subsp. <i>marinus</i> str. CCMP 1375	Pro_1316	Pro_1747	Pro_1330	No hits	Pro_1869

	<i>Synechococcus elongatus</i> PCC 6301	No hits	sync1055_d	sync1292_d <sup>†</sup>	No hits	sync1371_c sync1156_d
	<i>Thermo-synechococcus elongatus</i> BP-1	ttl1768	tlr1810	ttl1199 <sup>†</sup> ttl1767 <sup>†</sup> tlr0963 <sup>†</sup>	tlr1345	tlr2249 ttl2101
	<i>Synechocystis</i> sp. PCC 6803	sll1581	slr0328 slr0946	sll0923	sll5049	sll0737
	<i>Cyanobacterium</i> UCYN-A	No hits	No hits	UCYN_07970 <sup>†</sup>	No hits	UCYN_01240 UCYN_05370
	<i>Gloeobacter violaceus</i> PCC 7421	glr3784 gll3717 glr3239 gll2201 gll1797 glr0453	gll3174 glr0004	glr4310 glr3785 gll3716 gll2202 gll1796 glr0454	glr3855 gll3854 gll3709 gll1793 gll1781 glr0457	gll3744 glr2573 glr0819
Oscillatoriales	<i>Arthrospira platensis</i> NIES-39	NIES39_K02740 NIES39_M02090 NIES39_C03510 NIES39_C04930	NIES39_N00600 NIES39_L01410 NIES39_B01000	NIES39_C04940 NIES39_A06230 NIES39_C03520	NIES39_C04990	NIES39_C03330 NIES39_C04950 NIES39_O04190
	<i>Arthrospira maxima</i> CS-328	AmaxDRAFT_1209 AmaxDRAFT_2353 AmaxDRAFT_2896 AmaxDRAFT_4133	AmaxDRAFT_3417 AmaxDRAFT_1406	AmaxDRAFT_0719 AmaxDRAFT_2895 AmaxDRAFT_4132	AmaxDRAFT_2889	AmaxDRAFT_0674 AmaxDRAFT_4153

Continued

**Table 7.1** Putative cyanobacterial proteins involved in EPS assembly and export following the Wzy-dependent pathway —cont'd

Putative protein	Wza	Wzb	Wzc	Wzx	Wzy
InterPro*	IPR003715	IPR000106 IPR017867 IPR023485	IPR003856 IPR005702 IPR005701	IPR002797	IPR007016
<i>Lyngbya</i> sp. PCC 8106	L8106_15390 L8106_06289 L8106_06200 L8106_10507 L8106_14060 L8106_03112	L8106_20545 L8106_25130	L8106_15385 L8106_06284 L8106_09871 L8106_27951 L8106_10512 L8106_14065 L8106_03117	L8106_06309 L8106_09876 L8106_24490 L8106_19281 L8106_11602 L8106_08566	L8106_06389 L8106_09891 L8106_30415
<i>Oscillatoria</i> sp. PCC 6506	OSCI_1010013 OSCI_3300006 OSCI_3640002 OSCI_3640011	OSCI_420001 OSCI_810001	OSCI_1040007 OSCI_1010012 OSCI_3640004	OSCI_1010015	OSCI_1010014 OSCI_2040002 OSCI_3110005 OSCI_4130040
<i>Trichodesmium</i> <i>erythraeum</i> IMS101	Tery_2691 Tery_1924	Tery_4026 Tery_3848	Tery_1925	No hits	Tery_4577 Tery_3696
<i>Microcoleus</i> <i>chthonoplastes</i> PCC 7420	MC7420_2914 MC7420_5658 MC7420_6025 MC7420_917	MC7420_3589 MC7420_7127	MC7420_5618 MC7420_2223 MC7420_6195 MC7420_5066 MC7420_919	OSCI_1010015	MC7420_7720 MC7420_6228 MC7420_5038 MC7420_6656 MC7420_924

Nostocales	<i>Microcoleus vaginatus</i> FGP-2	MicvaDRAFT_4284 MicvaDRAFT_3852 MicvaDRAFT_1863 MicvaDRAFT_2067	MicvaDRAFT_2459 MicvaDRAFT_3619 MicvaDRAFT_3221	MicvaDRAFT_4285 MicvaDRAFT_2066 MicvaDRAFT_3627	MicvaDRAFT_4282 MicvaDRAFT_1953	MicvaDRAFT_4224 MicvaDRAFT_4283 MicvaDRAFT_3492
	<i>Nostoc azollae</i> 0708	Aazo_0827 Aazo_1061	Aazo_0818 Aazo_3363	Aazo_4516 Aazo_4925 Aazo_3125	No hits	Aazo_0822 Aazo_3351
	<i>Anabaena variabilis</i> ATCC 29413	Ava_3287 Ava_2909	Ava_2323 Ava_3712 Ava_3780 Ava_3461	Ava_4846 Ava_1386 Ava_1116 Ava_1045 Ava_0852 Ava_2908	Ava_4839 Ava_1384 Ava_1044 Ava_0846 Ava_0839	Ava_4842 Ava_3597 Ava_2327 Ava_1380 Ava_0851
	<i>Nodularia spumigena</i> CCY 9414	N9414_05784 N9414_23208	N9414_23168	N9414_07903 N9414_00965 N9414_07219 N9414_00005 N9414_07896 N9414_23213	No hits	N9414_23188 N9414_11129 N9414_00035
	<i>Nostoc puncti- forme</i> PCC 73102	Npun_R5507 Npun_F0458	Npun_F0449 Npun_F6486 Npun_F0951	Npun_R1070 Npun_F1381 Npun_R1496 Npun_R4851 Npun_R5250 Npun_F5505 Npun_F2453	Npun_R1494 Npun_R4850 Npun_F5499 Npun_F2442	Npun_F1371 Npun_R1492 Npun_R5097 Npun_F5501 Npun_R0453

Continued

**Table 7.1** Putative cyanobacterial proteins involved in EPS assembly and export following the Wzy-dependent pathway —cont'd

Putative protein	Wza	Wzb	Wzc	Wzx	Wzy
InterPro*	IPR003715	IPR000106 IPR017867 IPR023485	IPR003856 IPR005702 IPR005701	IPR002797	IPR007016
<i>Nostoc</i> sp. PCC 7120	all4388 alr2294 all0495	alr5068 alr1105 all3436 alr1067	all5222 all4432 alr3059 alr2856 alr2833 all0059 all0493	all4430 alr3072 alr3065 alr2857 all2290	all5073 all4428 alr3690 alr3060 alr2861
<i>Cylindrospermopsis raciborskii</i> CS-505	CRC_00854	CRC_02027	CRC_01492 CRC_01573 CRC_03354 CRC_03451	CRC_03341	CRC_01071 CRC_02023

\*InterPro conserved domains present on the proteins involved in well-characterized systems of bacterial EPS assembly and export that follow the Wzy-dependent pathways. The InterProScan Sequence Search (Hunter et al., 2012) was performed for the following proteins: Wza (AAD21562.1), BceE (ABD78147.1), AmsH (CAA54880.1), GumB (AAA86370.1); Wzb (AAD21563.1), BceD (ABD78146.1), AmsI (CAA54881.1), Wzc (AAD21564.1), BceF (ABD78148.1), AmsA (CAA54882.1), GumC (AAA86371.1), EpsA (NP\_053033.1), EpsB (NP\_053032.1), Wzx (AAD21572.1), Bcep1808\_4475 (YP\_001116905.1), AmsL (CAA54890.1), GumJ (AAA86378.1), EpsK (NP\_053023.1), Wzy (AAD21566.1), BceI (CAR54720.1), AmsC (CAA54884.1), GumE (AAA86373.1), EpsI (NP\_053025.1). The presence or absence of the domains identified was subsequently investigated in the selected cyanobacteria. Due to the involvement of IPR019554 (soluble ligand-binding domain; Wza) and IPR002586 (CobQ/CobB/MinD/ParA nucleotide-binding domain; Wzc) in other cellular processes, these domains were excluded from searches.

†Proteins identified by blast search homology using *E. coli* Wzc protein (AAD21564.1) as query. BlastP hits were considered significant when e-value was below or equal to  $e = 05$ .

**Table 7.2** Putative cyanobacterial proteins involved in EPS assembly and export following the ABC-dependent pathway

	Putative protein	KpsM	KpsC and KpsS	KpsU	KpsF	KpsE	KpsD
	InterPro*	IPR000412 IPR013525 IPR013526	IPR007833	IPR003329 IPR004528	IPR001347 IPR004800	IPR003856 IPR005705	IPR003715
Chroococcales	<i>Cyanothece</i> sp. CCY 0110	CY0110_22207 CY0110_23501 CY0110_28429 CY0110_29409 CY0110_29999	No hits	CY0110_05737	CY0110_07649 CY0110_06234 CY0110_27849	No hits	CY0110_03539 CY0110_28374
	<i>Cyanothece</i> sp. ATCC 51142	cce_2090 cce_4199 cce_1232 cce_1471 cce_1759	No hits	cce_0338	cce_2266 cce_1266 cce_0043	cce_1468	cce_4188 cce_1803
	<i>Cyanothece</i> sp. PCC 7822	Cyan7822_0258 Cyan7822_5345 Cyan7822_3051 Cyan7822_1204 Cyan7822_3729 Cyan7822_0158 Cyan7822_5719 Cyan7822_5786	No hits	No hits	Cyan7822_0343 Cyan7822_0820	Cyan7822_0712 Cyan7822_1903 Cyan7822_0908 Cyan7822_5718	Cyan7822_0713 Cyan7822_0909 Cyan7822_3789
	<i>Microcystis aeruginosa</i> NIES-843	MAE_21960 MAE_27940 MAE_42480 MAE_17300 MAE_17500	No hits	No hits	MAE_55680 MAE_27050 MAE_08000	MAE_32940 MAE_41520	MAE_37150 MAE_41410
	<i>Prochlorococcus marinus</i> str. MIT 9301	P9301_04741	No hits	P9301_14031 P9301_14131 P9301_14211	P9301_14341 P9301_17991	No hits	No hits

Continued

**Table 7.2** Putative cyanobacterial proteins involved in EPS assembly and export following the ABC-dependent pathway—cont'd

Putative protein	KpsM	KpsC and KpsS	KpsU	KpsF	KpsE	KpsD
InterPro*	IPR000412 IPR013525 IPR013526	IPR007833	IPR003329 IPR004528	IPR001347 IPR004800	IPR003856 IPR005705	IPR003715
<i>Prochlorococcus marinus</i> subsp. <i>marinus</i> str. CCMP 1375	Pro_0446	No hits	No hits	Pro_1766 Pro_1321 Pro_0941	Pro_1330	Pro_1316
<i>Synechococcus elongatus</i> PCC 6301	syc1943_c syc0423_d syc0246_c	No hits	syc1810_d	syc1809_d syc1533_d syc0987_d	No hits	No hits
<i>Thermosynechococcus elongatus</i> BP-1	tl11340 tl11329 tl11324 tlr1253	No hits	No hits	tl11237 tlr1171	No hits	Tll1768
<i>Synechocystis</i> sp. PCC 6803	slr0977 slr2107 sll0574 sll0760 sll0778	slr2115	slr2122	sll0220 sll0861 sll0083	sll0923	sll1581
<i>Cyanobacterium</i> UCYN-A	UCYN_02890 UCYN_09490	No hits	No hits	UCYN_03670 UCYN_08450 UCYN_12600	No hits	No hits
<i>Gloeobacter violaceus</i> PCC 7421	glr3473 gll3335 glr2168 glr2017 glr2016 gll0674	No hits	glr3775	glr4012 gll2215	glr4310 glr3785 gll3716 gll2202 gll1796 glr0454	glr3784 gll3717 glr3239 gll2201 gll1797 glr0453

Oscillatoriales	<i>Arthrospira platensis</i> NIES-39	NIES39_H00370 NIES39_J05740 NIES39_K00680 NIES39_B00480 NIES39_C03710 NIES39_D01320	No hits	NIES39_A00040	NIES39_Q02250 NIES39_G00550	NIES39_C04940	NIES39_K02740 NIES39_M02090 NIES39_C03510 NIES39_C04930
	<i>Arthrospira maxima</i> CS-328	AmaxDRAFT_0225 AmaxDRAFT_0326 AmaxDRAFT_1050 AmaxDRAFT_1469 AmaxDRAFT_3222	No hits	AmaxDRAFT_4373 AmaxDRAFT_4385	AmaxDRAFT_1377 AmaxDRAFT_2023	AmaxDRAFT_0719 AmaxDRAFT_2895	AmaxDRAFT_1209 AmaxDRAFT_2353 AmaxDRAFT_2896 AmaxDRAFT_4133
	<i>Lyngbya</i> sp. PCC 8106	L8106_05001 L8106_15400 L8106_24930 L8106_28511	No hits	L8106_13400	L8106_09586 L8106_12540 L8106_19878	L8106_15385 L8106_06284 L8106_09871 L8106_27951 L8106_10512 L8106_14065 L8106_03117	L8106_15390 L8106_06289 L8106_06200 L8106_10507 L8106_14060 L8106_03112
	<i>Oscillatoria</i> sp. PCC 6506	OSCI_270008 OSCI_940015 OSCI_1190002 OSCI_3460080 OSCI_3480008 OSCI_3720045 OSCI_4120005	No hits	No hits	OSCI_1490002 OSCI_3340001 OSCI_3370011	OSCI_1040007	OSCI_1010013 OSCI_3300006 OSCI_3640002 OSCI_3640011
	<i>Trichodesmium ery-</i> <i>thraeum</i> IMS101	Tery_1363 Tery_1079	No hits	No hits	Tery_3105 Tery_0455 Tery_0340	No hits	Tery_2691 Tery_1924
	<i>Microcoleus chthono-</i> <i>plastes</i> PCC 7420	MC7420_7226 MC7420_3065 MC7420_316 MC7420_4722 MC7420_5344 MC7420_2059 MC7420_6706 MC7420_6908	No hits	No hits	MC7420_7694 MC7420_1658	MC7420_5618 MC7420_2223 MC7420_6195 MC7420_5066 MC7420_919	MC7420_2914 MC7420_5658 MC7420_6025 MC7420_917

Continued



**Table 7.2** Putative cyanobacterial proteins involved in EPS assembly and export following the ABC-dependent pathway—cont'd

	Putative protein	KpsM	KpsC and KpsS	KpsU	KpsF	KpsE	KpsD
	InterPro*	IPR000412 IPR013525 IPR013526	IPR007833	IPR003329 IPR004528	IPR001347 IPR004800	IPR003856 IPR005705	IPR003715
Nostocales	<i>Microcoleus vaginatus</i> FGP-2	MicvaDRAFT_3831 MicvaDRAFT_2728 MicvaDRAFT_4920 MicvaDRAFT_0159	No hits	No hits	MicvaDRAFT_5363 MicvaDRAFT_3493	MicvaDRAFT_4285 MicvaDRAFT_2066	MicvaDRAFT_4284 MicvaDRAFT_3852 MicvaDRAFT_1863 MicvaDRAFT_2067
	<i>Nostoc azollae</i> 0708	Aazo_4903 Aazo_5199 Aazo_3861	No hits	No hits	Aazo_4573 Aazo_5218	Aazo_4516 Aazo_4925 Aazo_3125	Aazo_0827 Aazo_1061
	<i>Anabaena variabilis</i> ATCC 29413	Ava_4150 Ava_3346 Ava_1451 Ava_0687 Ava_0192	No hits	No hits	Ava_3485 Ava_0242	Ava_4846 Ava_1386 Ava_1116 Ava_1045 Ava_0852	Ava_3287 Ava_2909
	<i>Nodularia spumigena</i> CCY 9414	N9414_05030 N9414_17283 N9414_21691 N9414_07004 N9414_07933 N9414_01502	No hits	N9414_07943	N9414_05639 N9414_15757	N9414_07903 N9414_00965 N9414_07219 N9414_00005	N9414_05784 N9414_23208
	<i>Nostoc punctiforme</i> PCC 73102	Npun_F4532 Npun_F4672 Npun_F1934 Npun_F1935 Npun_F0491	Npun_R0678	Npun_R0682 Npun_R0688	Npun_R6016 Npun_F5214	Npun_R1070 Npun_F1381 Npun_R1496 Npun_R4851 Npun_R5250 Npun_F5505 Npun_F2453	Npun_R5507 Npun_F0458

<i>Nostoc</i> sp. PCC 7120	alr4485 all4219 alr2373 alr1500 alr1491 alr0076 all7196 all0917	No hits	No hits	alr3464 alr2432	all5222 all4432 alr3059 alr2856 alr2833 all0059	all4388 alr2294 all0495
<i>Cylindrospermopsis raciborskii</i> CS-505	CRC_00166 CRC_00787 CRC_01166 CRC_02438	CRC_03381	CRC_02913	CRC_00110 CRC_00115	CRC_01492 CRC_01573 CRC_03354 CRC_03451	CRC_00854

\*InterPro conserved domains present on the proteins involved in well-characterized systems of bacterial EPS assembly and export that follow the ABC-dependent pathways. The InterProScan Sequence Search (Hunter et al., 2012) was performed for the following proteins: KpsM (AAD31428.1), KpsM (CAE55827.1), NMA0196 (YP\_002341739.1), BexB (CAA38733.1), KpsC (AAD32183.1), KpsC (CAE55818.1), NMA0186 (YP\_002341732.1), KpsS (AAD32184.1), KpsS (CAE55819.1), NMA0185 (YP\_002341731.1), KpsU (CAE55817.1), KpsF (CAE55814.1), KpsE (AAD31430.1), KpsE (CAE55815.1), NMA0197 (YP\_002341740.1), BexC (CAA38732.1), KpsD (AAD31427.1), KpsD (CAE55816.1), NMA0198 (YP\_002341741.1), BexD (CAA38730.1). Due to the involvement of IPR000644 and IPR013785 (Cystathionine beta-synthase, core and Aldolase-type TIM barrel, respectively; KpsF), and IPR019554 (Soluble ligand binding domain; KpsD) in other cellular processes, these domains were excluded from searches. The characteristic InterPro domains of KpsT, the nucleotide-binding subunit of the ABC transporter, were not screened due to their presence in other ATPases components (Cuthbertson et al., 2010).

capsule export via the ABC-dependent pathway, with defects on these proteins leading to the accumulation of the polymer inside the cell (Cieslewicz & Vimr, 1996; Whitfield, 2006).

The pattern of the genes encoding the Wz\_ and Kps\_ homologues was consistent with that observed previous for cyanobacteria, with the gene copies scattered throughout the genomes, either isolated or in small clusters (Pereira *et al.*, 2009). It is possible that this pattern results from intragenomic duplication events and/or HGT. Given that gene duplication generates a broad adaptive potential, some of the copies may be related to specific phenotypes such as strains' morphology and ecological niches. Thus, it is important to unveil the evolutionary events that led to the pattern observed for these genes in cyanobacteria.

Due to the pivotal roles of PCP and OPX proteins in EPS assembly and export pathways, special attention was given to these proteins. The diversity and phylogenetic relationship of several Gram-negative PCP and OPX representatives were recently reviewed (Cuthbertson *et al.*, 2009). In general, the major phylogenetic groups defined for the PCP proteins matched those established for the correspondent OPX protein (present in the same gene cluster), likely reflecting their co-evolution. As a result, six groups of EPS assembly and export components were identified, where groups A, C, D, and E follow the Wz-dependent pathway and groups B and F participate in the ABC-dependent pathway. Within each group, the proteins from different bacteria display similar characteristics (high sequence similarity, domain organization, etc.). Although this study provided an important update in the knowledge of bacterial EPS assembly and export, cyanobacteria have not been contemplated (Cuthbertson *et al.*, 2009). To increase our understanding on cyanobacterial EPS assembly and export, the putative PCP and OPX identified were analysed from an evolutionary perspective. To achieve that, phylogenetic trees were computed using the Maximum Likelihood (ML) (Felsenstein, 1981) and the Neighbour-Joining (Saitou & Nei, 1987) algorithms, and compared to that obtained from the 16S rRNA. Regarding this last tree, the major clusters obtained are in agreement with the phylogenetic relationships described for cyanobacteria, determined using a single gene/protein or sequence concatenation methods (Larsson *et al.*, 2011; Swingley & Blankenship, 2008). In general, the clusters identified in the ML and NJ computed trees were consistent, strengthen the proposed model. The data gathered in this work provide the first insight in the evolutionary history of EPS-related genes in cyanobacteria. Most importantly, it defined new relevant questions and created the conditions for the design of new *in silico*,

genomics, transcriptomics and proteomics studies that will help clarify the evolutionary history of EPS assembly and export machinery in cyanobacteria.

### **3.1.1. Reconstruction of the PCP evolutionary history in cyanobacteria**

The members of the PCP protein family are characterized by having two transmembrane regions (TM1 and TM2, respectively) and the presence of a proline- and glycine-rich domain preceding and overlapping the TM2 (Cuthbertson et al., 2009; Morona et al., 2009). These features were consistently observed for the putative PCP identified in cyanobacteria (Table 7.3). Nevertheless, some of the selected unicellular cyanobacteria lacked an identifiable *pcp* homologue. Blast searches using *wzc* from *E. coli* group 1 capsule (K30) as query lead to the identification of three putative PCP in *Cyanothece* sp. PCC 0110 and *Thermosynechococcus elongatus* BP-1, and one putative PCP in *Synechococcus elongatus* PCC 6301 and *Cyanobacterium* UCYN-A. The analysis of the putative PCP proteins revealed that CY0110\_05934, Cyan7822\_0908, syc1292\_d, tlr0963 and UCYN\_07970 are considerably smaller, lacking the typical characteristics of PCP proteins. In addition, they are phylogenetically distant from all other cyanobacterial PCP proteins (Fig. 7.5, grey areas). Therefore, despite the sequence similarity shared with the Gram-negative PCP proteins, they are considerably different from their cyanobacterial counterparts, and therefore are likely to be involved in different cellular processes. Consequently, they were not considered for posterior analyses.

On the other hand, several other identified proteins are predicted to have additional transmembrane domains. These proteins appear intermixed with the other PCP homologues in bootstrapped-supported clusters, and thus are likely to be PCP homologues. In general, the cyanobacterial PCP show the typical features of the PCP-2a group, including the additional C-terminal region containing the Walker A and B domains, and the Dx/D motif commonly found in bacterial tyrosine kinases (Soulat et al., 2007). Nevertheless, some present minor deviations from the canonical sequences of these motifs, as was previously observed for other bacteria (Cuthbertson et al., 2009). Furthermore, the majority also possesses one or more C-terminal tyrosine residues.

The phylogenetic analysis of the cyanobacterial PCP proteins revealed the presence of 17 monophyletic clusters (1–17) and two larger bootstrapped-supported clusters (I and II). Most of the cyanobacterial sequences were grouped separately from the PCP proteins belonging to other bacteria, suggesting that after the early separation of the cyanobacterial branch, the genes encoding

Table 7.3 Characteristics of the putative PCP proteins identified for cyanobacteria

Organism	Locus tag	Length (no. aa)	Transmembrane domains*			P- and G-rich motif (preceding and overlapping TM2)	Walker A†		Walker B†	Y-rich C terminus (tail from Walker B)
			TM1	TM2	Other TMs		[A/G] XXXXGK[S/T]	DxD‡		
Chroococ- cales	<i>Cyanothece</i> sp. CCY 0110	CY0110_29394	754	34–54	464–484	Yes	AEPEDGCS	DTN	LVIYD	0
		CY0110_27525	736	17–37	436–456	Yes	VSNEEGKS	EGD	<b>MVIID</b>	0
		CY0110_05934	353			No	No	TTP	VPLLG	0
	<i>Cyanothece</i> sp. ATCC 51142	cce_1468	754	34–54	96–116 465–485	No	AESEDGCS	DTN	LVIYD	0
<i>Cyanothece</i> sp. PCC 7822	Cyan7822_0712 Cyan7822_1903 Cyan7822_0908 Cyan7822_5718		752	48–68	462–482	Yes	<b>ATAGEGKS</b>	EAN	LVIYD	0
			761	45–65	474–494	Yes	VQAQDGQS	DSN	LIYD	1
			475	24–44	438–458	Yes	AELFQNL	OLE	TPASP	0
			751	22–42	430–450	Yes	<b>ATTEEGKT</b>	DGN	YVLFD	0
<i>Microcystis aerugi- nosa</i> NIES-843	MAE_32940 MAE_41520		769	49–69	463–483 700–720	Yes	VSPGDGKT	<b>DAD</b>	LIYD	5
			705	22–42	433–453	Yes	SIFGEGKS	<b>DAD</b>	<b>FIID</b>	5
<i>Prochlorococcus marinus</i> subsp. <i>marinus</i> str. CCMP 1375	Pro_1330	585	38–58	429–449		Yes	FVAGCASA	SPK	FSVSL	0
<i>Synechococcus elongatus</i> PCC 6301	syc1292_d	252				No	No	MRP	VPVGA	0
<i>Thermosynechococ- cus elongatus</i> BP-1	tll1199 tll1767 tllr0963		786	42–62	488–508 599–619	Yes	TSGQEGQT	DAN	<b>VVIID</b>	1
			740	46–66	471–491	Yes	ALPGDGRT	<b>DGD</b>	<b>LVIVD</b>	1
			268			No	No	LRP	VPVAG	2
<i>Synechocystis</i> sp. PCC 6803	sll0923	756	40–60	135–155	711–731	No	<b>AESGDGKS</b>	<b>DGD</b>	<b>LILID</b>	8
<i>Cyanobacterium</i> UCYN-A	UCYN_07970	353				No	No	TTP	IPLLG	0

Oscillato- riales	<i>Gloeobacter violaceus</i>	glr4310	758	15–35	444–464	652–672	Yes	<u>ASPQEGKS</u>	<u>DGD</u>	<u>LVIFD</u>	5
	PCC 7421	glr3785	770	36–56	463–483		Yes	<u>SMPGEGKS</u>	<u>DAD</u>	<u>LVIID</u>	6
		gl13716	683	13–33	421–441		Yes	<u>STAGEGKT</u>	<u>DAD</u>	<u>YVLVD</u>	4
		gl12202	699	17–37	416–436		Yes	<u>AVANEGKS</u>	<u>DAD</u>	<u>QIIID</u>	4
		gl11796	676	13–33	420–440		Yes	<u>ANTGEGKT</u>	<u>DAD</u>	<u>YVLID</u>	3
		glr0454	676	13–33	421–441		Yes	<u>SGVGEGKT</u>	<u>DAD</u>	<u>YVLID</u>	4
	<i>Arthrospira platensis</i>	NIES39_C04940	740	27–47	432–452	468–488;	Yes	<u>SVPQEGKS</u>	<u>DGD</u>	<u>FVIID</u>	3
	NIES-39	NIES39_A06230	588	255–275	439–459	649–669	No	<u>SVPGEGKS</u>	<u>DSD</u>	<u>LVYYD</u>	2
		NIES39_C03520	721	37–57		552–572	Yes	<u>ARPADGKS</u>	<u>DAD</u>	<u>LVVYD</u>	3
	<i>Arthrospira maxima</i>	AmaxDRAFT_0719	743	27–47	408–428	469–489;	Yes	<u>SVPGEGKS</u>	<u>DSD</u>	<u>LVYYD</u>	2
	CS-328	AmaxDRAFT_2895	739	27–47	432–452	649–669	Yes	<u>SVPQEGKS</u>	<u>DGD</u>	<u>FVIID</u>	3
		AmaxDRAFT_4132	742	54–74	460–480	573–673	Yes	<u>ARPADGKS</u>	<u>DAD</u>	<u>LVYYD</u>	3
	<i>Lyngbya</i> sp. PCC	L8106_15385	751	52–72	430–450	516–536	Yes	<u>PSQSDGKS</u>	<u>DAN</u>	<u>WVIYD</u>	2
	8106	L8106_06284	744	52–72	459–479	636–656	Yes	<u>ATLGDGKS</u>	<u>DAD</u>	<u>LVYYD</u>	0
		L8106_09871	736	24–74	431–451	544–564	Yes	<u>AVSQEGKS</u>	<u>DAD</u>	<u>FIIVD</u>	3
		L8106_27951	741	28–48	436–456	653–673	Yes	<u>ALNGAGKS</u>	<u>DAN</u>	<u>LILLD</u>	2
		L8106_10512	754	36–56	423–443		Yes	<u>SQPGEKGS</u>	<u>DTD</u>	<u>LVYYD</u>	5
		L8106_14065	758	56–76	476–496		Yes	<u>ATPADGKS</u>	<u>DAD</u>	<u>LVYYD</u>	4
		L8106_03117	753		464–484		No	<u>AESGDGKS</u>	<u>DAN</u>	<u>LVYYD</u>	3
	<i>Oscillatoria</i> sp.	OSCI_1040007	774	51–71	474–494	588–608	Yes	<u>AAPGDGKS</u>	<u>DAN</u>	<u>LVYYD</u>	0
	PCC 6506	OSCI_1010012	782	51–71	461–481		Yes	<u>AVTGDGKS</u>	<u>DAD</u>	<u>LVYYD</u>	1
		OSCI_3640004	511	223–243			No	<u>CQVADGKS</u>	<u>DAD</u>	<u>LIYYD</u>	5
	<i>Trichodesmium</i>	Tery_1925	801	57–77	20–540	740–760	No	<u>ALPGDGKS</u>	<u>DAD</u>	<u>LVICD</u>	5
	<i>erythraeum</i>										
	IMS101										
<i>Microcoleus chthono- plastes</i> PCC 7420	MC7420_5618	784	51–71	459–479			Yes	<u>ATPSEGKS</u>	<u>DAD</u>	<u>LVYYD</u>	6
	MC7420_2223	765	50–70	463–483			Yes	<u>AVPADGKS</u>	<u>DAD</u>	<u>LVIFD</u>	6
	MC7420_6195	748	51–71	456–476			Yes	<u>PISGDGKT</u>	<u>DTN</u>	<u>FVIYYD</u>	2
	MC7420_5066	784	71–91	496–516			Yes	<u>AALGDGKS</u>	<u>DAN</u>	<u>LVVYD</u>	1
	MC7420_919	760	51–71	457–477			Yes	<u>AIPGDGKT</u>	<u>DTD</u>	<u>LVYYD</u>	2

Continued

Table 7.3 Characteristics of the putative PCP proteins identified for cyanobacteria—cont'd

			Transmembrane domains*			P- and G-rich motif (preceding and overlapping TM2)	Walker A <sup>†</sup>		Walker B <sup>‡</sup>		Y-rich C terminus (tail from Walker B)
Organism	Locus tag	Length (no. aa)	TM1	TM2	Other TMs		[A/G] XXXXGK[S/T]	DxD <sup>†</sup>	hhhhD <sup>‡</sup>		
Nostocales	<i>Microcoleus vaginatus</i> FGP-2	MicvaDRAFT_4285	778	52–72	457–477		Yes	<u>AVAGDGKS</u>	<u>DAD</u>	LVIYD	2
		MicvaDRAFT_2066	774	53–73	480–500		Yes	<u>CQVADGKS</u>	<u>DAD</u>	LVIYD	5
		MicvaDRAFT_3627	785	51–71	477–497		Yes	<u>ATVGEKGS</u>	DAN	LVIYD	2
	<i>Nostoc azollae</i> 0708	Aazo_4516	740	33–53	448–468		Yes	<u>ALPGEKGT</u>	DAN	<u>LVLID</u>	1
		Aazo_4925	739	53–73	430–450		Yes	TMPGDGKT	<u>DGD</u>	LVIYD	4
		Aazo_3125	743	30–50	448–468		Yes	<u>ALPDDGKS</u>	DAN	<u>LVLID</u>	2
	<i>Anabaena variabilis</i> ATCC 29413	Ava_4846	742	21–41	434–454	647–667	Yes	<u>ALPREGKS</u>	<u>DGD</u>	<u>FVIID</u>	1
		Ava_1386	734	25–45	431–451	527–547	Yes	SVPKEGKS	<u>DAD</u>	<u>FIIIFD</u>	3
		Ava_1116	741	31–51	443–463		Yes	PLLDRGKS	DAN	<u>LVIID</u>	3
		Ava_1045	736	24–44	432–452		Yes	CVMNEGKS	<u>DAD</u>	<u>YVIID</u>	4
Ava_0852 Ava_2908		727	16–36	426–446		Yes	PLAGEGKS	<u>DAD</u>	<u>LVIID</u>	4	
		727	16–36	430–450		Yes	TSSQEGKT	ETD	<u>LVLID</u>	2	
<i>Nodularia spumigena</i> CCY 9414	N9414_07903	304	50–70	445–46		No	No	No	No	No	
	N9414_00965	747	32–525	459–479		Yes	ALRDESKS	DAN	<u>LVLID</u>	2	
	N9414_07219	732	29–49	410–430		Yes	<u>ALSREGKT</u>	DAN	<u>LVIID</u>	0	
	N9414_00005	597	2–22	429–449		Yes	CLPGEGKS	<u>DAD</u>	No	No	
	N9414_07896	230	17–37			No	VMAGEGKS	<u>DGD</u>	LVIYD	4	
	N9414_23213	713				Yes	TSSQEGKT	ETD	<u>LVLID</u>	1	

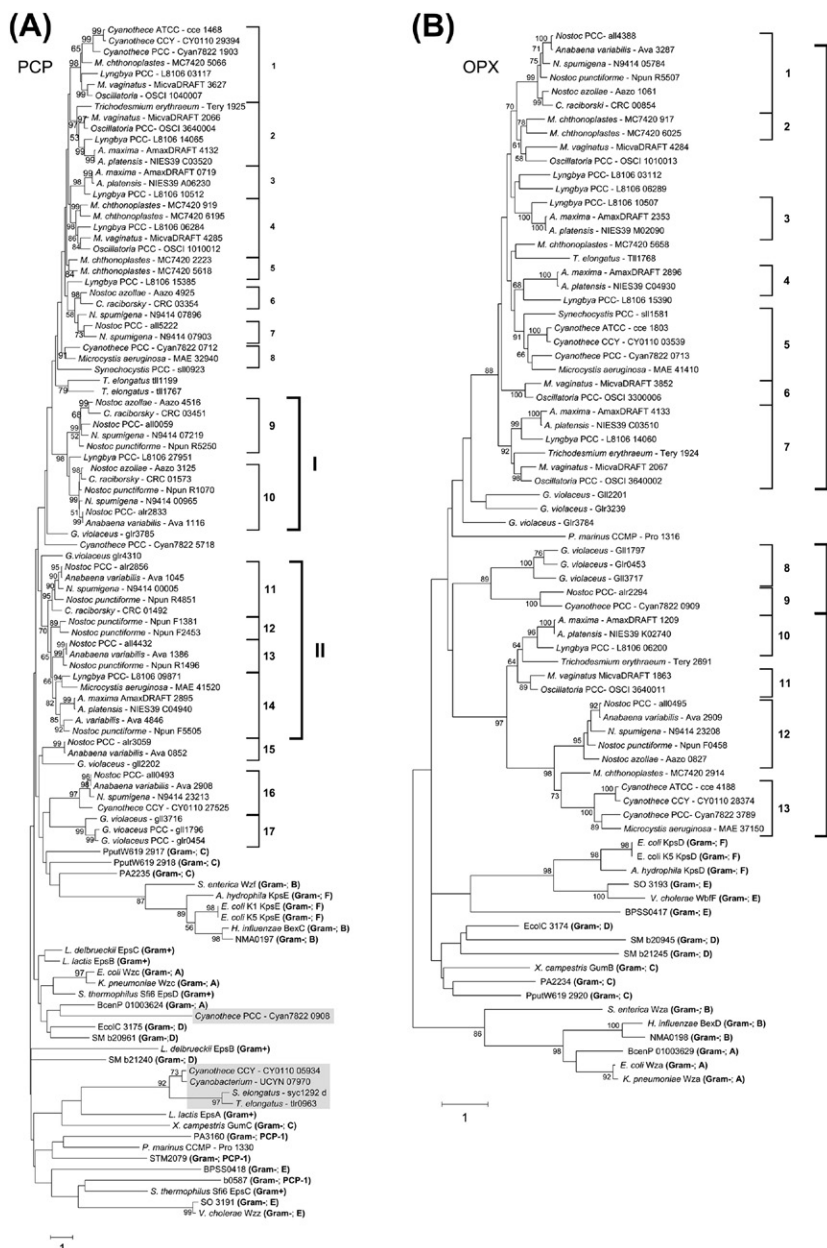
<i>Nostoc punctiforme</i> PCC 73102	Npun_R1070	742	30–50	430–450	649–669;	No	<b><u>ALPDEGKS</u></b>	DAN	<b><u>LVLID</u></b>	2
	Npun_F1381	700	21–41	431–451	683–703	Yes	<u>SVPREGKS</u>	DGN	<b><u>FVIID</u></b>	0
	Npun_R1496	734	25–45	431–451		Yes	SVGKEGKS	<b><u>DAD</u></b>	<b><u>LVIFD</u></b>	1
	Npun_R4851	736	22–42	457–477		Yes	STPGEGRS	<b><u>DAD</u></b>	<b><u>CVIID</u></b>	5
	Npun_R5250	738	35–55	442–462		Yes	<b><u>ALPGEGKT</u></b>	DAN	<b><u>LVLID</u></b>	0
	Npun_F5505	748	25–45	429–449		Yes	<b><u>AVPKEGKS</u></b>	<b><u>DAD</u></b>	<b><u>FVIID</u></b>	0
<i>Nostoc</i> sp. PCC 7120	Npun_F2453	467	22–42			No	No	No	No	No
	all5222	714	52–72	428–448	647–667	Yes	VMAGDGKS	DVN	LVIYD	2
	all4432	734	25–45	431–451	527–547	Yes	SVPKEGKS	<b><u>DAD</u></b>	<b><u>FIIFD</u></b>	3
	alr3059	727	16–36	426–446		Yes	PLSGEGKS	<b><u>DAD</u></b>	<b><u>LVIID</u></b>	4
	alr2856	736	24–44	432–452		Yes	CVMNEGKS	<b><u>DAD</u></b>	<b><u>CVIID</u></b>	4
	alr2833	771	61–81	473–493		Yes	PLLDRGKS	DAN	<b><u>LVIID</u></b>	3
	all0059	727	33–53	450–470		Yes	ALPGEGRT	DAN	<b><u>LVLID</u></b>	2
	all0493	727	16–36	430–450		Yes	TSSQEGKT	ETD	<b><u>LVILD</u></b>	2
<i>Cylindrospermop-</i> <i>sis raciborskii</i> <i>CS-505</i>	CRC_01492	746	41–61	449–469		Yes	SSPGEKKS	<b><u>DGD</u></b>	<b><u>FVIID</u></b>	7
	CRC_01573	741	31–51	443–463		Yes	ALPDISKs	DAN	<b><u>LVLID</u></b>	2
	CRC_03354	776	53–73	434–454		Yes	<b><u>ALKGEGKS</u></b>	<b><u>DTD</u></b>	<b><u>LVIYD</u></b>	7
	CRC_03451	724	24–44	434–454		Yes	<b><u>ALPGEGKT</u></b>	DGN	<b><u>LVIID</u></b>	0

\*The prediction of transmembrane domains was performed using TOPCONS (Bernsel, Viklund, Hennerdal, & Elofsson, 2009).

†The motifs in agreements with the canonical Walker A, DXD, and Walker B (Cuthbertson et al., 2009; Morona et al., 2000; Soulat et al., 2007; Walker et al., 1982) are in bold and underlined.

‡h: hydrophobic amino acid.





**Figure 7.5** Phylogenetic trees of cyanobacterial putative PCP (A) and OPX (B) proteins. The Maximum Likelihood phylogenetic trees were built in MEGA 5.05 (Tamura et al., 2011), using the WAG substitution model (Whelan & Goldman, 2001) with frequencies and a gamma-distributed rate of variation across sites. To assure statistic significance, 100 bootstraps were used in the computation of each tree – bootstrap values higher than

these proteins accumulated a significant amount of changes leading to a considerable phylogenetic distance. With few exceptions, the PCP homologues from the same strain were present in different clusters, while the orthologues from different organisms cluster together. These results suggest that they are not the outcome of gene duplication events within each strain's genome, but rather result of an earlier duplication/HGT event that occurred in a common ancestral organism. For unicellular cyanobacteria, only three orthologues were grouped in a cluster supported by a strong bootstrap value (cluster 1), together with a short number of sequences from filamentous strains. Regarding other cyanobacteria, a monophyletic cluster (cluster 2) comprising PCP orthologues from filamentous strains was defined, whereas three different consistent clusters comprising sequences from heterocystous strains could be observed (clusters 9–11). Clusters 9 and 10 are supported by a bootstrap value of 99% each. The synteny analysis of the genes encoding the PCP proteins from cluster 9 revealed that, with the exception of the *Nostoc* sp. PCC 7120 orthologue, these genes are contiguous to those encoding a SpoIID domain-containing protein. SpoIID are autolysins that hydrolyse the cell wall and drive membrane movement during sporulation (Meyer, Gutierrez, Pogliano, & Dworkin, 2010). In cyanobacteria, the role of these proteins is not clear although they do not seem to be essential for cell division in the unicellular cyanobacterium *Synechocystis*

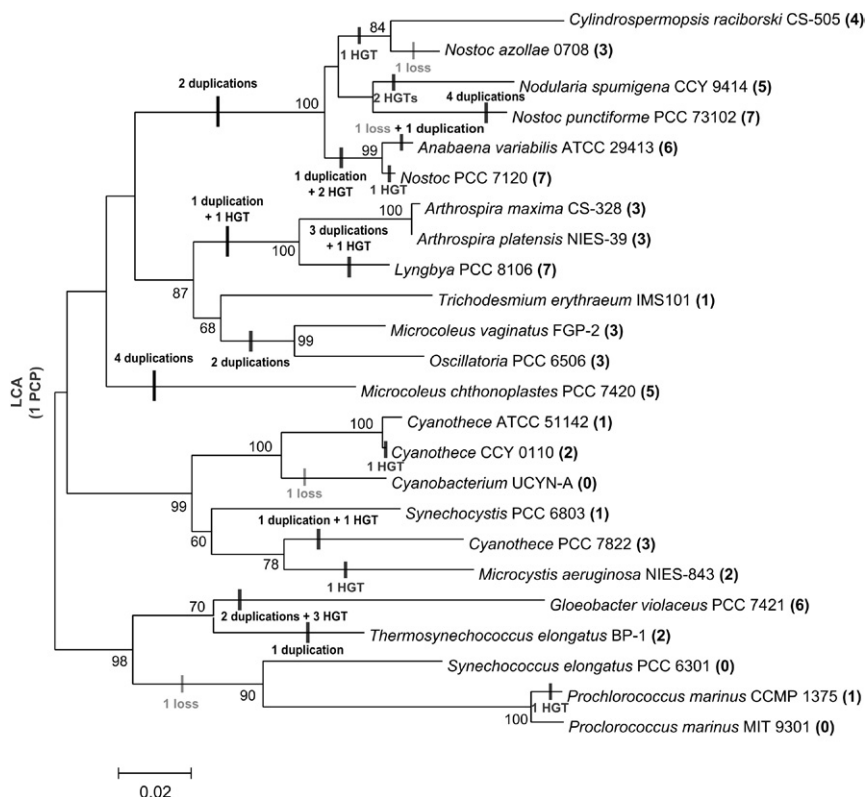
50% are indicated in the tree branches. Three representatives from each of the groups (A–F) defined in the study conducted by Cuthbertson et al. (2009) were selected as reference groups. Proteins from Gram-negative (Gram–) and Gram-positive bacteria are also indicated (Gram+). Bootstrapped supporter clusters are indicated in each tree (Arabic and Roman numbers). Grey background areas indicate putative PCP proteins identified by BLAST homology searches that are phylogenetically distant from other cyanobacterial homologues. Unicellular cyanobacteria: *Cyanothece* sp. CCY 0110 (*Cyanothece* CCY); *Cyanothece* sp. ATCC 51142 (*Cyanothece* ATCC); *Cyanothece* sp. PCC 7822 (*Cyanothece* PCC); *Microcystis aeruginosa* NIES-843 (*Microcystis aeruginosa*); *Prochlorococcus marinus* subsp. *marinus* str. CCMP 1375 (*P. marinus* CCMP); *Synechococcus elongatus* PCC 6301 (*S. elongatus*); *Thermosynechococcus elongatus* BP-1 (*T. elongatus*); *Synechocystis* sp. PCC 6803 (*Synechocystis* PCC); *Cyanobacterium* UCYN-A (*Cyanobacterium* UCYN\_A); *Gloeobacter violaceus* PCC 7421 (*G. violaceus*). Filamentous cyanobacteria: *Arthrospira platensis* NIES-39 (*A. platensis*); *Arthrospira maxima* CS-328 (*A. maxima*); *Lyngbya* sp. PCC 8106 (*Lyngbya* PCC); *Oscillatoria* sp. PCC 6506 (*Oscillatoria* PCC); *Trichodesmium erythraeum* IMS101 (*Trichodesmium erythraeum*); *Microcoleus chthonoplastes* PCC 7420 (*M. chthonoplastes*); *Microcoleus vaginatus* FGP-2 (*M. vaginatus*). Heterocystous cyanobacteria: *Nostoc azollae* 0708 (*Nostoc azollae*); *Anabaena variabilis* ATCC 29413 (*Anabaena variabilis*); *Nodularia spumigena* CCY 9414 (*N. spumigena*); *Nostoc punctiforme* PCC 73102 (*Nostoc punctiforme*); *Nostoc* sp. PCC 7120 (*Nostoc* PCC); *Cylindrospermopsis raciborskii* CS-505 (*C. raciborskii*).

PCC 6803 (Marbouty, Saguez, Cassier-Chauvat, & Chauvat, 2009). Interestingly, the closest homologue of several bacterial tyrosine kinases, including PCP proteins, is MinD. This protein is involved in bacterial cell division, allowing the correct localization of the Z-ring during cell division (Cuthbertson *et al.*, 2009). Thus, it is reasonable to hypothesize that the proteins comprised in cluster 9 may be involved in the cell division process. Similarly, the genes encoding the proteins from the cluster 10 are all in the vicinity of *hepA* (with the possible exception of N9414\_00965, whose genome sequence is not completely assembled yet). In addition, it is well established that, in *Nostoc* sp. PCC 7120, the gene cluster comprising the ORFs *alr2825*–*alr2841* is required for the production of the polysaccharidic layer of the heterocysts envelope (Holland & Wolk, 1990; Huang *et al.*, 2005; Xu, Elhai, & Wolk, 2008). Due to the synteny encountered for *alr2833* and its orthologues in cluster 10, it is likely that these PCP proteins also participate in this process. The involvement of PCP proteins in the production of the heterocysts polysaccharidic layer is not unexpected since previous studies have even suggested that the heterocyst envelope may itself be an LPS (Huang *et al.*, 2005). The high bootstrap value of the large cluster I, comprising clusters 9 and 10, points towards a paralogous duplication event in the last common ancestral organism of the heterocystous cyanobacteria, followed by fine-tuning of each paralogue according to the selective forces that acted upon them.

Altogether, the results obtained suggest the existence of a PCP-encoding gene in the last common ancestor of cyanobacteria (hereafter designated *pcp*), which underwent a series of duplication events, specific losses and HGT events, giving rise to the homologues comprised in clusters 1–17. Based on the analysis of the PCP phylogenetic tree, its comparison with the 16S rRNA tree computed here, and the principle of parsimony, it is possible to reconstruct the major evolutionary events that lead to the pattern observed nowadays (Fig. 7.6).

#### 3.1.1.1. Unicellular cyanobacteria

Considering the presence of a single *pcp* in the last common cyanobacterial ancestor, the ancestor of the cluster formed by *Synechococcus* and *Prochlorococcus* spp. lost its *pcp* orthologue. Subsequently, *Prochlorococcus marinus* CCMP 1375 acquired a *pcp* by HGT as supported by the phylogenetic distance from other cyanobacteria orthologues. On the other hand, *pcp* underwent a paralogous duplication in *Thermosynechococcus*, and two duplications in *Gloeobacter violaceus* PCC 7421. In addition, in the last strain, three HGT are likely to have occurred, giving rise to the sequences that compose cluster



**Figure 7.6** Proposed models for the putative PCP-encoding genes in cyanobacteria. The phylogenetic tree of the cyanobacterial 16S rRNA sequences was generated by Maximum Likelihood analysis in MEGA 5.05 (Tamura et al., 2011), using the Jukes–Cantor model (Jukes & Cantor, 1969) with a gamma-distributed rate of variation across sites. To assure statistic significance, 1000 bootstraps were used in the computation of each tree. The number of *pcp* homologues present in each organism is indicated in brackets. The predicted evolutionary events are also depicted. HGT: horizontal gene transfer; LCA: last common ancestor.

17 and the sequences that appear interspersed within the other cyanobacterial PCP homologues. After the divergence of *Cyanothece* ATCC 51142 and *Cyanothece* sp. CCY 0110, this last strain also acquired a second *pcp* by HGT, as supported by the high value of bootstrap (97%) of cluster 16, in which CY0110\_27525 is grouped with heterocystous sequences. In addition, the genomic context of the gene encoding this protein varies considerably compared to that of the *Cyanothece* ATCC 51142 and CCY 0110 sequences comprised in cluster 1. The *Cyanobacterium* UCYN-A possesses the smallest cyanobacterial genome known to date, and thus, the lack of

a PCP protein in this organism is most certainly the result from the loss of its ancestor orthologue during the contraction of its genome. Regarding *Microcystis aeruginosa* NIES-843, the significant sequence similarity of one of its sequences with PCP belonging to filamentous and heterocystous strains (cluster 14) suggests that its encoded gene was acquired by HGT. A similar origin is also predicted for the gene encoding the Cyan7822\_5718 in *Cyanothece* sp. PCC 7822, which is phylogenetically distant from other cyanobacterial PCP. In this organism, a paralogous duplication is predicted to have originated the third PCP protein.

### 3.1.1.2. Filamentous cyanobacteria

All the filamentous strains analysed possess one or more PCP protein. Cluster 2, which is supported by a high bootstrap value (97%), comprises one orthologue from each of these strains, with the exception of *Microcoleus chthonoplastes* PCC 7420. The genomic context of the genes encoding these proteins is relatively conserved, indicating a common origin (Guerrero *et al.*, 2005). The absence of an orthologue from *Microcoleus* is not surprising as this organism branched early within this group, being distantly related from the other filamentous strains. After its separation from the other strains, four duplications within its genome lead to the five paralogues identified. A few other paralogous duplications can be postulated for the filamentous strains: two occurring in the common ancestor of *Microcoleus vaginatus* FGP-2 and *Oscillatoria* PCC 6506 (clusters 1 and 4), another occurring in the lineage that gave rise to *Arthrospira maxima* CS-328, *Arthrospira platensis* NIES-39, and *Lyngbya* PCC 8106 (cluster 3), and three occurring in this last organism after its separation from the *Arthrospira* spp. ancestor (sequences comprised in clusters 1 and 4 and L8106\_15385). The presence of L8106\_09871, AmaxDRAFT\_2895 and NIES39\_C04940 in cluster II, along with sequences from heterocystous cyanobacteria, strongly suggests an HGT event occurring in the last common ancestor of these three filamentous strains. Indeed, the genomic regions containing the genes encoding AmaxDRAFT\_2895 and NIES39\_C04940 are very similar, supporting the hypothesis of a common origin for these genes in these strains. In addition, although the synteny does not extend to *Lyngbya*, L8106\_09871 is phylogenetically close to the *Arthrospira* spp. orthologues, suggesting a reshuffle of this gene within *Lyngbya*'s genome. An additional HGT event, occurring after the branching of this strain can account for the gene encoding the L8106\_27951, grouped with heterocystous sequences in cluster I.

### 3.1.1.3. Heterocystous cyanobacteria

In general, the *pcp* homologues from heterocystous cyanobacteria are grouped within clusters supported by high bootstrap values, similar to that observed for 16S rRNA sequences (Brito et al., 2012). As previously mentioned, two duplication events are predicted to have occurred in the last common ancestor of the heterocystous strains, giving rise to the paralogues putatively involved in cell division (cluster 9) or in the synthesis of the polysaccharidic layer of heterocysts (cluster 10). Interestingly, *Anabaena variabilis* ATCC 29413 appears to have lost its *pcp* orthologue from cluster 9. A *pcp* orthologue from the obligate symbiont *Nostoc azollae* 0708 is also missing from cluster 11. The loss of this orthologue may be related with its symbiotic competence. Nevertheless, one additional PCP sequence of this strain and one belonging to *C. raciborskii* CS-505 are grouped in a cluster separated from other heterocystous sequences (cluster 6), most likely resulting from the acquisition of a *pcp* by their common ancestor through HGT. A paralogous duplication is also expected to have occurred in the ancestor of *A. variabilis* ATCC 29413 and *Nostoc* PCC 7120 (cluster 13). In addition, this ancestor also acquired two *pcp* sequences by HGT, giving rise to the sequences grouped in clusters 15 and 16. After the branching of these organisms, a paralogous duplication gave rise to the *A. variabilis* sequence in cluster 14, whereas *Nostoc* acquired another homologue by HGT (cluster 7). Regarding *N. punctiforme* PCC 73102, four duplications are postulated, given that all of these paralogues cluster together with sequences from heterocystous cyanobacteria. A different pattern is observed for *Nodularia spumigina* CCY 9414, where two HGT events gave rise to the homologue from cluster 7 (the sequences encoding N9414\_07896 and N9414\_07903 are in different genomic contigs, but are most likely part of the same gene) and that of cluster 16. It is interesting to observe that this cluster comprises sequences that resulted from two separate events of HGT in heterocystous strains, namely, in the ancestor of *A. variabilis* and *N. punctiforme* and in *Nodularia*. Given the strong phylogenetic relationship usually observed for heterocystous strains, it is possible that these particular sequences play an important physiological role in these strains.

### 3.1.2. Reconstruction of the OPX evolutionary history in cyanobacteria

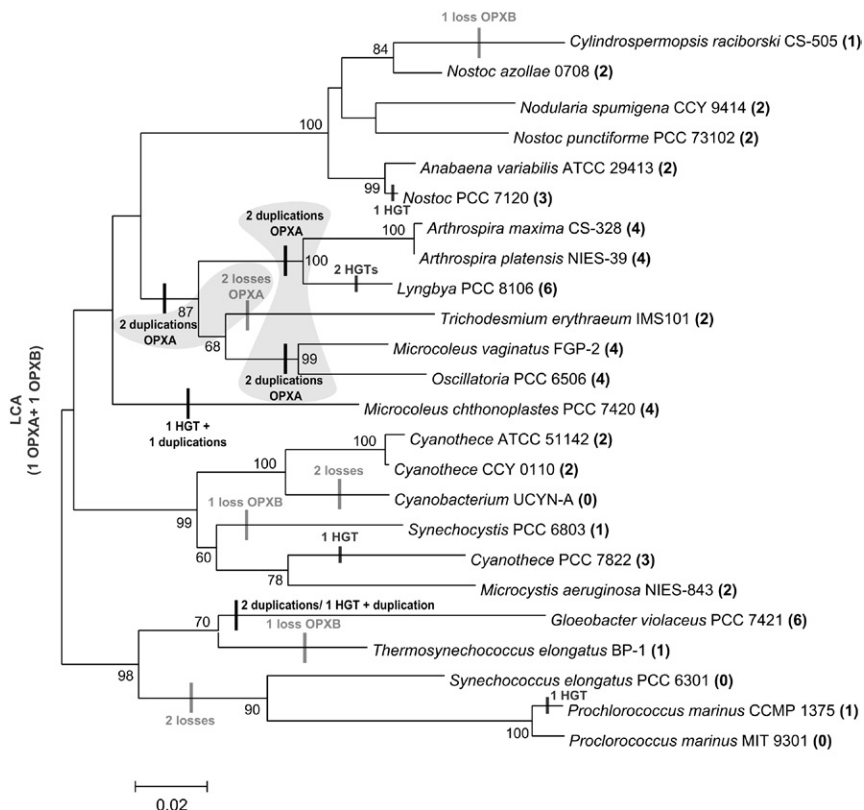
The OPX proteins are characterized by the presence of a PES domain (IPR003715). The analysis performed revealed that this motif is common in cyanobacteria, with most of the strains possessing at least one putative

OPX homologue. Exceptions were observed for only three unicellular cyanobacteria, namely, *P. marinus* str. MIT 9301, *S. elongatus* PCC 6301 and *Cyanobacterium* UCYN-A. Most of the putative OPX identified also possess a soluble ligand-binding beta-grasp domain (SLBB; IPR019554). This domain is typical of proteins that contain a beta-grasp, and it has been hypothesized that it may play a role in soluble or small-molecule ligand recognition. SLBB is found not only among bacterial polysaccharide export proteins, but also in other proteins, such as the competence DNA receptor ComEA and the Nqo1 subunit of the respiratory electron transport chain (Burroughs, Balaji, Iyer, & Aravind, 2007). Regarding the cyanobacterial OPX proteins, 13 monophyletic clusters (1–13) were observed (Fig. 7.5B). Similar to that observed for PCP, the OPX proteins belonging to each strain were also grouped in different clusters, emphasizing the role of duplication events in the evolution of the genes encoding OPX proteins (hereafter referred to as *opx*). In general, the clusters are consistent with the morphologies displayed by the strains, with clusters 5 and 13 including OPX sequences from unicellular strains, clusters 2, 3, 4, 6, and 11 comprising the OPX representatives from filamentous cyanobacteria, and clusters 1 and 12 including the sequences of the heterocystous strains (Fig. 7.5B). In addition, three larger clusters could also be distinguished (I–III), with clusters I and III comprising at least one bootstrap-supported cluster associated with each morphological group (Fig. 7.5B). These results strongly suggest that the last common cyanobacterial ancestor possessed two *opx* paralogues, here designated as *opxA* and *opxB*, which underwent a series of duplication events, specific losses and HGT, giving rise to the homologues comprised in clusters I and III, respectively (Fig. 7.7).

### 3.1.2.1. Unicellular cyanobacteria

According to the hypothesis of the presence of two paralogues, *opxA* and *opxB*, in the last common ancestor of cyanobacteria, it is likely that these two genes were lost in the lineage that gave rise to the monophyletic cluster composed by *Synechococcus* and *Prochlorococcus* spp. (Fig. 7.7). Later on, *P. marinus* CCMP 1375 acquired an *opx* gene by HGT, being the encoded protein phylogenetically separated from other cyanobacterial OPX. On the other hand, both *Thermosynechococcus* and *Synechocystis* lost the *opxB* in two separate events. The *Gloeobacter* OPX sequences comprised in cluster 8 show a high degree of sequence similarity, being likely the result of two paralogous duplications from one of the ancestor *opx*. The other putative OPX probably originated by an HGT followed by duplication. The fact that none





**Figure 7.7** Proposed models for the putative OPX-encoding genes in cyanobacteria. The phylogenetic tree of the cyanobacterial 16S rRNA sequences was generated by Maximum-likelihood analysis in MEGA 5.05 (Tamura et al., 2011), using the Jukes–Cantor model (Jukes & Cantor, 1969) with a gamma-distributed rate of variation across sites. To assure statistic significance, 1000 bootstraps were used in the computation of each tree. The number of *opx* homologues present in each organism is indicated in brackets. The predicted evolutionary events are also depicted. HGT: horizontal gene transfer; LCA: last common ancestor.

of the *Gloeobacter* sequences is included in clusters I and III (related to the *opxA* and *opxB* ancestor paralogues, respectively) may be related with the early branching of *Gloeotheca* within the radiation of cyanobacteria, which resulted in the accumulation of numerous mutations in the *opx* genes. This hypothesis is also coherent with the works reporting that this strain possesses unique characteristics within the cyanobacterial phylum (Nakamura et al., 2003). Similar to the events predicted for *pcp*, the *Cyanobacterium* also lost the two ancestor *opx* paralogues during the contraction of its genome. Interestingly, *Cyanothece* PCC 7822 possesses three OPX. One of the



proteins is grouped in cluster I, being encoded by *opxA*. A second protein is comprised in cluster II, being codified by *opxB*. Finally, Cyan7822\_0909 forms a cluster with a bootstrap value of 100% with alr2294 from *Nostoc* sp. 7120. This unexpected phylogenetic proximity is likely the result of HGT. Interestingly, in what concerns the unicellular cyanobacteria, only the *opxA* and *opxB* of *Cyanothece* sp. PCC 7822 and *Microcystis* are located in the vicinity of *pcp* homologues although the PCP encoded by the genes close to *opxB* do not possess the typical Interpro domains of other bacterial PCP proteins, which prevented its identification.

### 3.1.2.2. Filamentous cyanobacteria

Most of the filamentous strains possess more than two *opx* homologues. The phylogenetic relationship observed for the OPX from cluster 7 is supported by a high bootstrap value. In addition, the genes encoding these proteins are contiguous to *pcp* orthologues, whose encoded proteins are also consistently grouped in the PCP tree (Fig. 7.5A, cluster 2). This strongly suggests that the cluster 7 OPX proteins are the outcome of the ancestor *opxA* and that, later on, two paralogous duplications occurred in the lineage that give rise to *Lyngbya* and *Arthrospira* spp., and in the last common ancestral organism of *Microcoleus* and *Oscillatoria*. Another parsimonious hypothesis should also be considered. Indeed, it is possible that the *opxA* gene underwent two paralogous duplications in the last common ancestor of the filamentous strains and, after speciation, *Trichodesmium* lost two of its paralogues. Although this last hypothesis is plausible, the distribution pattern of the OPX in the phylogenetic tree, with the orthologues of *Arthrospira* spp. being grouped separately from the ones belonging to *Microcoleus* and *Oscillatoria* strongly support the first scenario. The two additional OPX present in *Lyngbya* were probably acquired by HGT from other cyanobacteria, or result from an HGT followed by a paralogous duplication. Finally, for *M. chthonoplastes*, it is likely that *opxA* underwent a duplication event giving rise to the sequences grouped in cluster 2, whereas the MC7420\_5658 was probably acquired by HGT. All of these events gave rise to the large number of homologues from filamentous cyanobacteria that are comprised in cluster I. Regarding the cluster II, the *opxB* encoding the proteins comprised in clusters 10 and 11 are in the vicinity of genes putatively related to LPS biosynthesis. Nevertheless, similar to what was observed previously, these proteins do not possess the typical Interpro domains of other bacterial PCP proteins, and therefore, were not considered in this study.

### 3.1.2.3. Heterocystous cyanobacteria

The majority of the heterocystous strains analysed only possess two OPX, suggesting that the selective forces acting upon the *opx* homologues from heterocystous cyanobacteria were considerably different from the ones acting on those present in unicellular and filamentous strains. In fact, only two events are predicted to have occurred: the loss of *opxB* in *Cylindrospermopsis* and the acquisition of an additional *opx* in *Nostoc* sp. 7120 by HGT (cluster 9). Interestingly, the proteins comprised in cluster 12, encoded by *opxB* orthologues, possess two PES domains. The *opxB* encoding the all0495 from *Nostoc* sp. 7120, Ava\_2909 from *A. variabilis*, and N9414\_23208 from *Nodularia*, are contiguous to *pcp* homologues acquired by these organisms by HGT (Fig. 7.5A, cluster 16; see above, section 3.1.1.3.). The genes encoding the other OPX homologues grouped in cluster 12 are also in the vicinity of putative *pcp*, although they do not possess the typical Interpro domains of other bacterial PCP proteins. Consequently, it is possible that the acquisition of the *pcp* homologues by *Nostoc* sp. 7120, *A. variabilis* and *Nodularia* is related to the need of a *pcp* counterpart. Differently, the majority of *opxA* orthologues from heterocystous cyanobacteria (cluster 1) is located in the vicinity of a gene encoding an ABC transporter-like protein, with only Npun\_R5507 being close to the *pcp* encoded protein Npun\_F5505. Although this genomic context could suggest the involvement of these proteins in an ABC-dependent pathway, ABC transporters participate in numerous metabolic processes and none of these ABC transporter-like proteins possesses the characteristic domains of KpsM.



## 4. CONCLUDING REMARKS AND FUTURE PERSPECTIVES

Overall, the phylogenetic analyses conducted for the PCP and OPX unveiled a complex evolutionary history, characterized by specific losses, HGT events and numerous paralogous duplications. This scenario is consistent with previous reports sustaining that gene duplication is the main force of genome evolution in microorganisms (Hooper & Berg, 2003), generating diversity in terms of genetic resources that will then be selected and fine-tuned according to various adaptive pressures. Regarding the cyanobacterial *pcp*-encoded proteins, the evidences gathered suggest that they are abundant in the cyanobacterial genomes, particularly in heterocystous strains, where they might be involved in other processes such as the synthesis of the polysaccharidic layer of the heterocysts. In addition, most of the cyanobacteria

possess additional genes encoding proteins that may play similar functions, despite lacking the typical motifs generally found in PCP, as it was unveiled by the synteny analysis performed here. The differences anticipated for the mechanisms of EPS assembly and export in cyanobacteria may be related to its unique cellular envelope that combines features of both Gram-negative and Gram-positive bacteria (Hoiczky & Hansel, 2000), and/or to the complexity of the polysaccharidic polymers produced. Concerning the *opx*, the selective forces that acted upon them were different, favouring, in general, the loss of the *opxB* and the duplication of *opxA*. Interestingly, despite the lower number of *pcp* and *opx* copies present on the smaller cyanobacterial genomes, including those of *Prochlorococcus* spp., *Synechococcus* and *Thermosynechococcus*, no straightforward correlation between genome size and copy number is observed. The number of gene copies rather seems to be correlated with the morphological group, with the heterocystous strains possessing more *pcp* genes, whereas *opx* are generally more abundant in the filamentous cyanobacteria. Despite this general trend, *Gloeobacter* and *Lyngbya* possess a high number of homologues of both genes, and thus, it is probable that during evolution, these two strains were exposed to particular environmental conditions that selected the maintenance of this large number. No direct correlation was also observed between the number/phylogenetic relationships of *opx* and *pcp* genes with the strains' habitat and diazotrophic capacity. For instance, the same number of *pcp* homologues was found in the thermophilic *Thermosynechococcus*, the freshwater *Microcystis*, and the marine *Cyanothece* sp. CCY 0110. Similarly, the same number of *opx* was present in the non-N<sub>2</sub> fixing *Microcystis* and the diazotrophic *N. punctiforme*. Furthermore, no correlation was found with the ability to produce efficiently EPS. This outcome is not unexpected, since most cyanobacteria produce some type of EPS in the form of a well-defined sheath, a thick capsule, mucilage or released polysaccharides (RPS), but it remains to be known whether all of these structures are assembled following the same pathway. *Synechococcus elongatus* constitutes a fascinating case since, although it lacks identifiable PCP and OPX proteins and never forms a well-defined sheath, produces mucilage (Castenholz, 2001).

As more genome sequences and detailed genome annotations become available, more information can be used to complement these findings. The work presented here contributes to a better understanding of the evolution of the EPS assembly and export machinery in cyanobacteria, pinpointing specific evolutionary events and probable functions. However, there is still a long way to go in order to have a robust reconstruction of the evolution

history EPS-related genes in cyanobacteria, which harbour a greater complexity than their counterparts in other bacteria, therefore, hampering their complete characterization. The integration of this knowledge with data obtained from genomics, transcriptomics and proteomics studies will help to clarify the process of EPS production in cyanobacteria.

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# Gene Expression during Heterocyst Differentiation

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## Abstract

Some cyanobacteria (oxygenic phototrophs) grow as chains of vegetative cells (filaments or trichomes). When placed in media lacking combined nitrogen, some cells in the filaments differentiate into  $N_2$ -fixing heterocysts. The nitrogen fixation system is inactivated by oxygen, and the heterocyst provides a micro-oxic environment for nitrogenase to function. In this review, we first describe the special envelope and metabolism that makes the heterocyst micro-oxic, to concentrate then on the regulation of gene expression during the process of differentiation. Differentiation starts as a response to a persistent high cellular carbon-to-nitrogen balance signalled by 2-oxoglutarate, which results in activation of the global transcriptional regulator NtcA followed by increased expression, mainly localized to differentiating cells, of the *hetR* (encoding the differentiation-specific transcription factor HetR) and *ntcA* genes. The expression of genes encoding proteins that transform the vegetative cell into a heterocyst is then activated with a spatiotemporal specificity to produce a mature functional heterocyst. Recent global analyses have added information on time course and levels of gene expression during the process of differentiation, and much information is also available on the promoters of a number of these genes. Contiguous promoters building complex promoter regions are common among heterocyst-related genes. Understanding the molecular mechanism of operation of these promoters, including the roles of HetR and NtcA, is a major goal of research in this field.



## 1. INTRODUCTION

Filamentous cyanobacteria are organisms that grow as filaments or trichomes formed by strings of contiguous cells. The trichome is the organismic unit in these bacteria and mainly consists of vegetative cells that perform oxygenic photosynthesis (Rippka, Deruelles, Waterbury, Herdman, & Stanier, 1979). In filamentous cyanobacteria such as those of the genera *Anabaena* and *Nostoc*, some cells in the filament can differentiate into specialized cells with different functions (Flores & Herrero, 2010). Heterocysts are differentiated cells specialized in the fixation of atmospheric nitrogen ( $N_2$ ) that do not perform oxygenic photosynthesis. During the differentiation process, a specific program of gene expression is activated to produce proteins that convert the vegetative cell into a heterocyst, where specific genes that encode the nitrogen fixation machinery and the needed accompanying metabolic enzymes are expressed (Xu, Elhai, & Wolk, 2008). In the developed filament bearing the two cell types, vegetative cells and heterocysts exchange metabolites, with heterocysts providing the vegetative cells with fixed nitrogen and vegetative cells providing the heterocysts with photosynthate (Wolk, Ernst, & Elhai, 1994). Thus, the diazotrophic trichome is a truly multicellular organism that requires the activity of two interdependent cell

types for growth. In this review, we will first summarize the biochemical and morphological properties of the heterocyst to then focus on the program of gene expression that support the process of differentiation and its regulation.

There are numerous different filamentous cyanobacteria that can produce heterocysts, and the classical taxonomic study of Roger Y. Stanier and co-workers performed on pure cultures of cyanobacteria recognizes eight genera of heterocyst-forming cyanobacteria that belong to taxonomic Sections IV and V (Rippka et al., 1979). Genera of Sections IV and V are distinguished by cell division in one plane (perpendicular to the longitudinal axis of the filament) or in more than one plane, respectively. In taxonomic Section IV, a distinction is made between genera comprising strains that produce (such as *Nostoc*) or do not produce (such as *Anabaena*) hormogonia, which are small filaments frequently made of small cells that serve a dispersal function. Additionally, in different genera, the heterocysts may be found exclusively at the trichome ends or may be produced also intercalary, the latter being the case in strains of *Anabaena* and *Nostoc*. Although filamentation likely was an early evolutionary trait in cyanobacteria (Schirromeister, Antonelli, & Bagheri, 2011), heterocyst-forming cyanobacteria form a monophyletic group (Giovannoni et al., 1988). This makes the study of a few model organisms appropriate to delineate the basic aspects of heterocyst biology, which has been investigated mostly in strains of two genera belonging to Section IV, *Anabaena* and *Nostoc*. In cultures of strains of *Anabaena* and *Nostoc*, after nitrogen stepdown, heterocysts develop at semiregular intervals along the filament; in diazotrophic cultures, as vegetative cells grow and divide, heterocysts differentiate from vegetative cells located midway between existing heterocysts. The heterocysts are terminally differentiated cells that are supposed to die after a few generations of vegetative cells (Haselkorn, 2009).

Whereas the physiology and biochemistry of the heterocyst were initially worked out mainly in *Anabaena cylindrica* and *Anabaena variabilis*, molecular genetics approaches have been used mostly with *Anabaena* sp. strain PCC 7120 and *Nostoc punctiforme* strain PCC 73102 (ATCC 29133). The latter strains can receive plasmids by conjugation from *Escherichia coli* (Flores & Wolk, 1985; Wolk, Vonshak, Kehoe, & Elhai, 1984), which makes them amenable to genetic analysis (Cohen, Wallis, Campbell, & Meeks, 1994; Wolk et al., 1988), and the complete sequence of their genomes has been available for more than 10 years already (Kaneko et al., 2001 [<http://genome.kazusa.or.jp/cyanobase/Anabaena>]; Meeks, Campbell, Summers, & Wong, 2002 [<http://genome.jgi-psf.org/nospu/nospu.home.html>]). Although the

genomic sequence of *A. variabilis* has also been recently made available (<http://genome.jgi-psf.org/anava/anava.home.html>), and DNA can also be transferred to this strain (Zahalak, Pratte, Werth, & Thiel, 2004), it has been used in the genetic analysis of heterocyst differentiation less extensively than *Anabaena* sp. strain PCC 7120 or *Nostoc punctiforme*. *Anabaena* sp. strain PCC 7120 is also frequently referred to as *Nostoc* sp. but because the production of hormogonia in this strain is not obvious and its genomic sequence is highly similar to that of *Anabaena variabilis* but much less to that of *Nostoc punctiforme*, we will keep denoting it as *Anabaena* sp. strain PCC 7120. Although *Anabaena* sp. strain PCC 7120 has been more extensively used in genetic analysis than *Nostoc punctiforme*, both organisms have been subjected to transposon mutagenesis permitting the identification of many heterocyst differentiation genes (Cohen *et al.*, 1994; Wolk, Cai, & Panoff, 1991) and have been used in global transcriptomic analyses providing wide information on gene expression during heterocyst differentiation or in the mature heterocyst (Campbell, Summers, Christman, Martin, & Meeks, 2007; Christman *et al.*, 2011; Ehira & Ohmori, 2006a; Flaherty, van Nieuwerburgh, Head, & Golden, 2011; Mitschke *et al.*, 2011, see section 3.5 below).



## 2. THE HETEROCYST

### 2.1. The Heterocysts are Sites of Nitrogen Fixation

Heterocyst-forming cyanobacteria have been known for over 200 years (Rippka *et al.*, 1979), but the role of the heterocysts in the biology of these organisms remained elusive for a long time, to the point that heterocysts were considered ‘a botanical enigma’ (Fritsch, 1951). Because heterocyst production negatively correlates with the availability of combined nitrogen (Fogg, 1949) and there is a relationship between the presence of heterocysts and the capability to fix atmospheric nitrogen, Fay, Stewart, Walsby, and Fogg (1968) asked whether the heterocysts are the sites of nitrogen fixation in the filaments.

Nitrogen fixation is carried out in different bacteria by a strongly conserved enzyme complex, nitrogenase, which carries out the reduction of  $N_2$  producing two molecules of ammonia in a reaction that requires reductant and energy in the form of ATP (Rubio & Ludden, 2008). Nitrogenase is made of the dimeric Fe protein (nitrogenase reductase) and the tetrameric,  $\alpha_2\beta_2$ , Fe–Mo protein (properly, nitrogenase) that bears the unique metal cofactor FeMoCo (Rubio & Ludden, 2008). The nitrogenase complex is

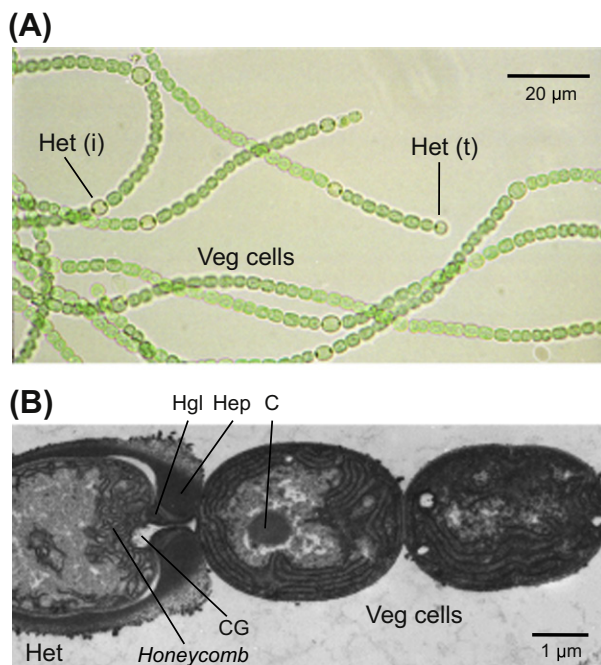
the product of the genes *nifH*, which encodes the Fe protein, and *nifD* and *nifK*, which encode the  $\alpha$  and  $\beta$  subunits of the Fe–Mo protein, respectively. These genes are generally found forming an operon, *nifHDK*, which is clustered together with other genes that are needed for the biosynthesis of FeMoCo and for the maturation of nitrogenase. An important feature of nitrogenase, which determines much of the biology of nitrogen fixation, is that it is extremely sensitive to oxygen, i.e. the enzyme is quickly and irreversibly inactivated by oxygen.

The hypothesis that nitrogenase resides in the heterocysts received strong support when a substantial fraction of the nitrogenase activity and of the nitrogenase proteins of diazotrophically grown filaments of *Anabaena variabilis* could be recovered in the heterocysts isolated from those filaments (Peterson & Wolk, 1978a; see also Fleming & Haselkorn, 1973). The presence of the Fe protein of nitrogenase in the heterocysts was confirmed by immunoelectron microscopic localization (Bergman, Lindblad, & Rai, 1986; Murry, Hallenbeck, & Benemann, 1984). Final support for the heterocysts as sites of nitrogen fixation came from studies using transcriptional fusions to the luciferase *luxAB* genes, in which transcription from the promoter of the *nifHDK* genes was observed confined to heterocysts (Elhai & Wolk, 1990). The heterocyst provides an appropriate environment for expression and function of the oxygen-labile nitrogenase. As described in the following sections, two aspects of the heterocyst are relevant for its function as a site of nitrogen fixation: its special cell envelope and its special metabolism.

## 2.2. The Heterocyst Envelope

### 2.2.1. Chemical nature

The heterocyst can be distinguished from the vegetative cells of the filament by its thick cell envelope and the presence of internal refractile polar bodies, which can be appreciated by light microscopy (Fig. 8.1A). Electron microscopy showed that a special cell envelope is present outside of the cell wall (meaning the cell wall also found in the vegetative cells) and that this envelope consists of two components, a dense inner layer and a less dense outer layer (Wildon & Mercer, 1963) (Fig. 8.1B). These two layers were further defined as an inner laminated layer on one hand, and as middle homogeneous and outermost fibrous layers on the other hand (Lang & Fay, 1971). The isolation of heterocyst cell walls permitted to perform a chemical analysis that showed carbohydrates (62%) and lipids (15%) as the major components of these walls (Dunn & Wolk, 1970). Indeed, glycolipids that are specific to heterocyst-forming cyanobacteria



**Figure 8.1** Parts of filaments of *Anabaena* sp. strain PCC 7120 observed by light microscopy (A) or transmission electron microscopy after chemical fixation (B), which was performed as described by Merino-Puerto *et al.* (2011). Het, heterocyst, which may be placed intercalary (i) or terminally (t) in the filament; Veg cells, vegetative cells; Hgl, heterocyst glycolipid layer; Hep, heterocyst envelope polysaccharide layer; C, carboxysome; *honeycomb*, heterocyst intracellular membrane system; CG, place where the cyanophycin granule (lost during sample preparation) was located. See the colour plate.

(Nichols & Wood, 1968) and that are present in heterocysts (Walsby & Nichols, 1969; Wolk & Simon, 1969) were identified. These heterocyst-specific glycolipids (Hgl) were found to constitute the laminated layer of the heterocyst envelope (Winklenbach, Wolk, & Jost, 1972) and their chemical structure has been determined (see Gambacorta, Trincone, Soriente, & Sodano, 1999; and references therein). They are glycosides of long chain triols, tetrols and hydroxyketones, with some variations in structure being found in different strains. The length of the glycolipid is about 3.5–4 nm (Winklenbach *et al.*, 1972) but the periodicity of the laminated layer in situ may be about 7–8 nm (reviewed in Wolk, 1982). Enrico Schleiff and co-workers have recently represented the unit of the glycolipid layer as a bilayer of inverted glycolipids (see Fig. 2 in Nicolaisen, Hahn, & Schleiff, 2009a). One or more glycolipid layer units may surround the whole heterocyst,

except at its poles, which are surrounded by many more layers (Fig. 8.1B; see Fig. 3A in Moslavac et al., 2007).

The homogeneous layer is largely composed of polysaccharide (Dunn & Wolk, 1970), and the outermost fibrous layer that is sometimes observed in electron micrographs of heterocysts has been suggested to represent a less compacted material of the same polysaccharide (Wolk, 1982). The chemical structure of the heterocyst polysaccharide (Hep) has been determined for a few heterocyst-forming cyanobacteria and mainly consists, as described for *Anabaena cylindrica*, of a  $\beta$ -1,3-linked mannosyl-glucosyl-glucosyl-glucose tetrasaccharide backbone to which side branches of mannose, glucose and glucosyl glucose, galactose, and xylose are attached (Cardemil & Wolk, 1979).

### 2.2.2. Biosynthesis and deposition

To understand the processes of production and deposition of the heterocyst envelope-specific Hgl and Hep layers, it is important to note that cyanobacteria are didermic bacteria in which a peptidoglycan layer and an outer membrane are found outside of the cytoplasmic membrane (Stanier & Cohen-Bazire, 1977; Wolk, 1973). As stated above, the heterocyst envelope is external to the cell wall also found in the vegetative cells (Lang & Fay, 1971; Wildon & Mercer, 1963; see also Fig. 2e in Flores, Herrero, Wolk, & Maldener, 2006). Genetic analysis of diazotrophic growth has identified many mutants that exhibit a  $\text{Fox}^-$  phenotype (requiring fixed nitrogen for growth in the presence of oxygen; see Lechno-Yossef, Fan, Wojciuch, & Wolk, 2011). Many of these mutants bear inactivated *hgl* or *hep* genes, which encode proteins needed for production of the Hgl and Hep layers, respectively, and specifically lack the corresponding layer. Notably, many *hgl* genes (Fan et al., 2005) on one hand and *hep* genes (Huang et al., 2005) on the other hand are clustered together in 'gene islands' in the genome of *Anabaena* sp. strain PCC 7120, although related genes are found in other chromosomal locations as well (Maldener, Hannus, & Kammerer, 2003; Wang et al., 2007).

In *Anabaena* sp. strain PCC 7120, a gene island including ORFs *all5343*–*alr5357* bears a number of *hgl* genes, whose mutation arrest production of Hgl (Fan et al., 2005). These genes encode putative fatty acid synthases and polyketide synthases that may account for the biosynthesis of the aglycone of Hgl. This gene island may extend to *all5341*, which has been shown to encode the putative glycosyl transferase (HglT) required to produce the principal Hgl of *Anabaena* sp. strain PCC 7120 (Awai & Wolk, 2007). A second set of *Anabaena* mutants synthesize Hgl detectable by thin layer chromatography but do not produce the Hgl layer detectable by electron



microscopy. The corresponding genes appear to encode proteins needed for Hgl deposition in the heterocyst envelope. Some such genes are found in the Hgl island and have been named *hgd* genes for Hgl deposition (Fan *et al.*, 2005), but some others are found in different chromosome locations. These include the genes of the *devBCA* operon, which encodes an ABC-type exporter (Fiedler, Arnold, Hannus, & Maldener, 1998), and *hgdD*, which encodes an outer membrane TolC-like protein (Moslavac *et al.*, 2007). DevBCA and HgdD can form a protein complex that traverse the cell wall and has been shown to constitute an ATP-driven exporter of Hgl (Staron, Forchhammer, & Maldener, 2011). Which role the other identified *hgd* genes, *hgdA*, *hgdB*, and *hgdC* (Fan *et al.*, 2005), may have in Hgl deposition remains to be established, as is also the case for HglK, a pentapeptide repeat-containing protein that bears four transmembrane segments and is necessary for localization of Hgl in the cell envelope (Black, Buikema, & Haselkorn, 1995).

The first heterocyst differentiation gene that was identified, *hetA* (Holland & Wolk, 1990), is involved in production of the heterocyst envelope polysaccharide layer (Wolk *et al.*, 1988) and, therefore, has been renamed *hepA* (Wolk, Elhai, Kuritz, & Holland, 1993). This gene is part of the *hep* gene island that includes from ORF *alr2822* to ORF *alr2841* of the genome of *Anabaena* sp. strain PCC 7120 (Huang *et al.*, 2005). HepA is an ABC-type transporter homologous to the *E. coli* exporter of the Lipid A of lipopolysaccharide (LPS). Other genes encoding enzymes including glycosyl transferases and additional genes encoding proteins homologous to LPS biosynthesis proteins are present in the *hep* gene island. These observations made C. Peter Wolk and co-workers ask whether Hep may be a particular type of LPS (Huang *et al.*, 2005). Hep genes in other locations of the chromosome include genes encoding other glycosyl transferases (Maldener *et al.*, 2003; Wang *et al.*, 2007) and *all4388* encoding a putative Wza-like periplasmic/outer membrane polysaccharide export protein (Lechno-Yossef *et al.*, 2011; Maldener *et al.*, 2003). *Escherichia coli* Wza is involved in the passage of the group 1 capsular polysaccharide through the cell envelope and represents a new paradigm in outer membrane proteins (Collins & Derrick, 2007). Thus, a number of genes encoding Hep biosynthesis and export proteins have been identified, but a final description of the process leading to production of Hep is pending.

### 2.2.3. Function

In cultures of different heterocyst-forming cyanobacteria incubated in the absence of combined nitrogen under anoxic conditions, heterocyst



differentiation is arrested at an early stage, and the corresponding filaments exhibit a nitrogenase activity that is very sensitive to oxygen (Rippka & Stanier, 1978). These proheterocysts lack a mature envelope suggesting a role of the heterocyst-specific envelope in preventing the entry of air into the heterocyst cytoplasm. Production of oxygen-sensitive nitrogenase was further correlated with lack of production of Hgl in mutants of *Anabaena variabilis* (Haury & Wolk, 1978). Analysis of mutants of *Anabaena* sp. strain PCC 7120 has shown that the barrier to oxygen requires both of the heterocyst envelope layers (Murry & Wolk, 1989). Because mutants lacking Hgl retain their Hep layer in heterocysts (Black et al., 1995; Fan et al., 2005) but mutants lacking Hep tend to show a delamination or fragmentation of the Hgl layer (Wolk, 2000), the idea, based on the possible low permeability of Hgl to oxygen and nitrogen (Walsby, 1985), that the Hgl layer is the principal barrier to gas diffusion into the heterocyst, whereas the Hep layer protects the Hgl layer from damage and dilution into the surrounding medium is widely accepted (Xu et al., 2008). Walsby (1985) suggested that the heterocyst envelope might provide the right degree of gas permeability to allow the entry of sufficient nitrogen gas for nitrogenase function and maintain an adequate micro-oxic environment to avoid nitrogenase inactivation. However, he has recently proposed that the main gas diffusion pathway into the heterocyst is through the cell envelope pores present at the vegetative cell–heterocyst junctions (Walsby, 2007). In any case, the heterocyst envelope appears to have the role of limiting the entry of air, including poisoning oxygen, into the heterocyst.

### 2.3. The Heterocyst Cytoplasm and Heterocyst Metabolism

Related to its activity of nitrogen fixation, the heterocyst has several notable metabolic properties including lack of oxygenic photosynthesis and photosynthetic carbon fixation, increased respiratory activity and nitrogen assimilation functions.

#### 2.3.1. Carbon metabolism

The heterocysts are less pigmented than the vegetative cells (Fay et al., 1968) and bear low levels of some photosystem II-related pigments (Thomas, 1970). Although they have some photosystem II components (Braun-Howland & Nierzwieki-Bauer, 1990) or even photosystem II complexes (Cardona et al., 2009), there is ample evidence that heterocysts lack the O<sub>2</sub>-evolving activity of photosystem II while keeping photosystem I activity (see, e.g. Almon & Böhme, 1980; Donze, Haveman, & Schiereck, 1972; Tel-Or &

Stewart, 1977). Lack of water photolysis would contribute to keeping a micro-oxic intracellular environment for nitrogenase but pays the price of loss of photosynthetic CO<sub>2</sub> fixation (Wolk, 1968). Indeed, heterocysts lack ribulose-*bis*-phosphate carboxylase/oxygenase (Rubisco) activity (Winklenbach & Wolk, 1973) and protein (see, e.g. Fig. 4 in Cardona *et al.*, 2009). Nitrogen fixation requires reductant and energy in the form of ATP (Rubio & Ludden, 2008). Carbon fixed in the vegetative cells moves to the heterocysts (Wolk, 1968), and sucrose has been long supposed to be the transferred organic substrate (Wolk *et al.*, 1994). Recent results have shown that a heterocyst-specific invertase is indeed needed for diazotrophic growth corroborating the idea that sucrose is a transferred substrate (López-Igual, Flores, & Herrero, 2010; Vargas, Nishi, Giarrocco, & Salerno, 2011). Sugar catabolism in the heterocyst follows the oxidative pentose phosphate pathway as suggested by detection of increased levels of glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase (Lex & Carr, 1974; Winklenbach & Wolk, 1973). Further, a requirement of glucose 6-phosphate dehydrogenase for nitrogen fixation has been evidenced by inactivation of the *zwf* gene in *Nostoc punctiforme* (Summers, Wallis, Campbell, & Meeks, 1995). Other substrates, such as the amino acid alanine, can also be transferred from vegetative cells to heterocysts, where alanine can be catabolized by alanine dehydrogenase and its products enter the tricarboxylic acid pathway (which in cyanobacteria lacks 2-oxoglutarate dehydrogenase) providing reductant (Jüttner, 1983; Pernil, Herrero, & Flores, 2010).

### 2.3.2. Bioenergetics

A distinct aspect of heterocyst bioenergetics is that the action spectrum of nitrogen fixation (measured by the acetylene reduction assay) corresponds to that of photosystem I activity (Fay, 1970). Because photosystem I-dependent cyclic electron flow takes place in the heterocysts (Almon & Böhme, 1982), cyclic photophosphorylation is likely important for nitrogen fixation. On the other hand, the reducing power in the form of NADPH produced in the oxidation of the sugar transferred from vegetative cells can be used for N<sub>2</sub> fixation via ferredoxin-NADP<sup>+</sup> oxidoreductase and the heterocyst-specific ferredoxin, FdxH, that in its reduced state can be a direct substrate of nitrogenase reductase (Masepohl, Schölisch, Görlitz, Kutzki, & Böhme, 1997; Razquin *et al.*, 1995, 1996). However, NADPH (and NADH) can also feed electrons into the electron transport chain of heterocyst membranes to provide photosystem I with electrons (that may serve in part for N<sub>2</sub> fixation; Lockau, Peterson, Wolk, & Burris, 1978) and reduce O<sub>2</sub>

to water by heterocyst-specific terminal respiratory oxidases. In *Anabaena* sp. strain PCC 7120, two gene clusters for haeme-copper oxidases, *cox2* (encoding a cytochrome *c* oxidase) and *cox3* (encoding a possible quinol oxidase), are expressed during heterocyst differentiation (Jones & Haselkorn, 2002; Valladares, Herrero, Pils, Schmetterer, & Flores, 2003; Valladares, Maldener, Muro-Pastor, Flores, & Herrero, 2007). Electrons from H<sub>2</sub>, produced as a byproduct in the nitrogenase reaction (Rubio & Ludden, 2008), can also follow these paths with the concurrence of an uptake hydrogenase (Happe, Schütz, & Böhme, 2000; Peterson & Wollk, 1978b). In addition to the membrane quinone pool and the soluble cytochrome *c*<sub>6</sub>, the electron transport chain involves the activities of the NAD(P)H dehydrogenase (NDH-1) and cytochrome *b*<sub>6</sub>-*f* complexes, in which, as is also the case for cytochrome *c* oxidase, energy conservation in the form of a proton gradient takes place. As is well known, the proton gradient results in ATP biosynthesis catalysed by the H<sup>+</sup>-transporting F<sub>0</sub>F<sub>1</sub> ATP synthase. The heterocysts contain a special arrangement of intracellular membranes that is located next to the heterocyst neck(s) and has been called the 'honeycomb' (Lang & Fay, 1971) (see Fig. 8.1B). Some cytochemical evidence obtained with *Anabaena cylindrica* suggests that respiration can take place in the heterocyst honeycomb (Murry, Olafsen, & Benemann, 1981), and a recent proteomic analysis of heterocyst membranes from *Nostoc punctiforme* has found that the intracellular membranes are dominated by photosystem I and ATPase proteins, whereas the NDH-1 and cytochrome *b*<sub>6</sub>-*f* complexes are readily identified in a 'cell wall' fraction that contains cytoplasmic membranes (Cardona et al., 2009). Subcellular localization of the bioenergetic complexes of the heterocyst will merit further investigation.

### 2.3.3. Nitrogen metabolism

As described above, the heterocyst contains nitrogenase and is the site of nitrogen fixation in filaments grown under oxic conditions. The nitrogenase produces ammonia that, as shown by fixation of <sup>13</sup>N-labelled N<sub>2</sub> by whole filaments, is immediately incorporated as the amido group of glutamine, from which the label passes to the amino group of glutamate (Wolk, Thomas, Shaffer, Austin, & Galonsky, 1976). This pattern of labelling is consistent with incorporation of ammonia through the glutamine synthetase-glutamate synthase (GS/GOGAT) cyclic pathway, in which ammonia is added to glutamate by the ATP-dependent glutamine synthetase and the resulting glutamine transfers its amido group to 2-oxoglutarate producing two molecules of glutamate in a reaction catalysed by glutamate synthase, which requires reductant

that in many cyanobacteria is provided by reduced ferredoxin (reviewed in Luque & Forchhammer, 2008). The heterocyst contains high levels of glutamine synthetase (Dharmawardene, Haystead, & Stewart, 1973; Thomas, Meeks, Wolk, Shaffer, & Austin, 1977), which respond to a specific pattern of expression of the *glnA* gene encoding this enzyme (Tumer, Robinson, & Haselkorn, 1983; Valladares, Muro-Pastor, Herrero, & Flores, 2004), but lacks glutamate synthase (Martín-Figueroa, Navarro, & Florencio, 2000; Thomas *et al.*, 1977). Additionally, heterocysts isolated from *Anabaena cylindrica* can produce glutamine from glutamate and ammonia (Thomas *et al.*, 1977). These observations suggested that an exchange of glutamine for glutamate takes place between heterocysts and vegetative cells, in which heterocysts provide the vegetative cells with fixed nitrogen in the form of glutamine and the vegetative cells provide the heterocysts with the glutamate needed for the glutamine synthetase reaction (Thomas *et al.*, 1977; Wolk *et al.*, 1976).

Not all the nitrogen fixed by the heterocyst is immediately exported. The heterocysts conspicuously bear refractile polar granules in the neck regions that form in the cell poles proximal to adjacent vegetative cells (Fig. 8.1A). These polar granules are made of cyanophycin (Lang, Simon, & Wolk, 1972), which is multi-L-arginyl-poly-(L-aspartic acid), a nitrogen reserve that accumulates after nitrogenase activity peaks late in differentiation (Sherman, Tucker, & Sherman, 2000). However, the accumulation of cyanophycin is not required for diazotrophic growth as demonstrated by inactivation of cyanophycin synthetase(s) in both *Anabaena variabilis* (Ziegler, Stephan, Pistorius, Ruppel, & Lockau, 2001) and *Anabaena* sp. strain PCC 7120 (Picossi, Valladares, Flores, & Herrero, 2004). On the other hand, excessive accumulation of cyanophycin resulting from inactivation of cyanophycinase, the first enzyme acting in cyanophycin degradation, impairs diazotrophic growth (Picossi *et al.*, 2004).

## 2.4. Intercellular Molecular Exchange

As described above, the heterocyst provides the vegetative cells in the filament with fixed nitrogen, and glutamine is a likely nitrogen vehicle (Thomas *et al.*, 1977; Wolk *et al.*, 1976). Additionally, because the heterocyst cannot perform the photosynthetic fixation of CO<sub>2</sub>, it needs to be fed with reduced carbon, and sucrose is likely received from the photosynthetic vegetative cells as a source of reductant and energy (López-Igual *et al.*, 2010; Vargas *et al.*, 2011). Additionally, glutamate (Thomas *et al.*, 1977) and alanine (Pernil *et al.*, 2010) are likely transferred from vegetative cells to heterocysts.

As it will be described, in addition to these metabolites, some regulators appear to be transferred between cells in the cyanobacterial filament. Possible paths of transfer are starting to be understood.

The outer membrane of the cell envelope is continuous along the filament in heterocyst-forming cyanobacteria (see Flores et al., 2006; Wilk et al., 2011) defining a continuous periplasm that can constitute a communication path between the cells in the filament (Mariscal, Herrero, & Flores, 2007). In favour of the view that the periplasm is a communication conduit, the outer membrane has been found to be relatively impermeable to metabolites such as sucrose and glutamate that are important in the diazotrophic physiology (Nicolaisen et al., 2009b). In this scenario, transporters mediating the transfer of substrates between the periplasm and the cytoplasm would be important for diazotrophy. Such transporters are known at least for some amino acids (Picossi et al., 2005). Additionally, at least two types of protein complexes appear to link directly adjacent cells in the filament, those containing SepJ and those containing FraC/D (Merino-Puerto et al., 2011). All these proteins have been found to reside in the intercellular septa along the filament (Flores et al., 2007; Merino-Puerto, Mariscal, Mullineaux, Herrero, & Flores, 2010) and to be required for the intercellular transfer of some fluorescent tracers (Merino-Puerto et al., 2010, 2011; Mullineaux et al., 2008). The structures containing these proteins might correspond to those termed *microplasmodesmata* that have been observed by conventional transmission electron microscopy (Lang & Fay, 1971) and by freeze-fracture electron microscopy (Giddings & Staehelin, 1978), and that have been recently suggested to be named *septosomes* after their observation by electron tomography (Wilk et al., 2011). Which metabolites and regulators are transferred through each of these paths (the periplasm, the SepJ-containing and the FraC/D-containing septal complexes) remain to be established.



### 3. THE SPECIFIC PROGRAM OF GENE EXPRESSION

#### 3.1. Triggering of the Differentiation Process

As mentioned above, the presence of heterocysts in a cyanobacterial filament negatively correlates with the availability of combined nitrogen. Consistently, nitrogen starvation is an environmental cue that determines heterocyst differentiation. The impact of nitrogen starvation on metabolism depends, however, on the availability of other nutrients, notably sources of carbon. Thus, when carbon is readily available, nitrogen starvation results in a high carbon-to-nitrogen balance in the cell, whereas for limiting carbon, the impact of

nitrogen starvation is lower (see, e.g. Vázquez-Bermúdez, Paz-Yepes, Herrero & Flores, 2002b). Because cyanobacteria lack 2-oxoglutarate dehydrogenase (Smith, London, & Stanier, 1967), the main fate of 2-oxoglutarate in these organisms is incorporation into glutamate and glutamine (Vázquez-Bermúdez, Herrero, & Flores, 2000). Under conditions of sufficient fixation of CO<sub>2</sub>, nitrogen deprivation restricts the use of 2-oxoglutarate resulting in its accumulation in the cells, which has been suggested to indicate a high cellular carbon-to-nitrogen balance (Muro-Pastor, Reyes, & Florencio, 2001). Consistently, addition of 2-oxoglutarate to a unicellular cyanobacterium bearing a heterologous 2-oxoglutarate permease parallels the effect of a high carbon supply in the expression of some nitrogen assimilation genes (Vázquez-Bermúdez, Herrero, & Flores, 2003). A similar approach showed that 2-oxoglutarate and a nonmetabolizable analogue of it, 2,2-difluoropentanedioic acid, can promote heterocyst differentiation in *Anabaena* sp. strain PCC 7120 (Laurent *et al.*, 2005; Li, Laurent, Konde, Bédu, & Zhang, 2003). These results are consistent with the notion that a high cellular carbon-to-nitrogen balance signalled by 2-oxoglutarate triggers heterocyst differentiation. The molecular basis of this effect relies on the role of 2-oxoglutarate as an effector of the transcription factor NtcA, which will be described below.

Another protein sensing 2-oxoglutarate levels in cyanobacteria is P<sub>II</sub>, the *glnB* gene product, which is phosphorylated under conditions determining a high carbon-to-nitrogen balance in the cells (Forchhammer, 2008). In *Anabaena* sp. strain PCC 7120, the *glnB* gene is repressed in the heterocysts (Paz-Yepes, Flores, & Herrero, 2009), which nonetheless appear to contain some nonphosphorylated P<sub>II</sub> protein (Laurent *et al.*, 2004). An *Anabaena* mutant of this gene could be obtained only after overexpression of downstream genes (Paz-Yepes *et al.*, 2009). This mutant was impaired specifically in diazotrophic growth but could produce heterocysts that lacked polar (cyanophycin) granules, a phenotype similar to that of an *Anabaena* mutant impaired in dephosphorylation of the P<sub>II</sub> protein (Laurent *et al.*, 2004). As mentioned above, cyanophycin contains arginine, and promotion of arginine biosynthesis is the best-known function of the P<sub>II</sub> protein in its dephosphorylated state (Forchhammer, 2008). The relevance of this P<sub>II</sub> effect on heterocyst biology remains to be assessed.

### 3.2. Genes Activated Transiently during Differentiation with Spatial Specificity

Heterocyst differentiation is the result of a specific program of gene expression that is established as a last response to the external cue of nitrogen

deprivation, exhibiting both temporal and spatial components of specificity. When the organism senses nitrogen deficiency, likely as an increase in the carbon-to-nitrogen balance in the cells, an early response consists in the activation of genes for the scavenging of traces of combined nitrogen in the form of ammonium, nitrate, nitrite or urea (Luque & Forchhammer, 2008; see section 3.5 below). If nitrogen deficiency persists, heterocyst differentiation starts and proceeds involving the sequential action of the products of a number of regulatory and structural genes. Many genes involved in heterocyst differentiation have been identified after the isolation of mutants unable to grow fixing  $N_2$  under oxic conditions and the study of their phenotypes, and recently by global studies.

Genes responsible for heterocyst differentiation are activated in a sequential manner. Traditionally, three rough categories of genes have been distinguished according to the time of induction during the differentiation process: early genes, activated at c. 3–4 h after combined nitrogen step-down, which include the *hetR* regulatory gene; intermediate genes including those that are activated at c. 6–8 h, such as *ntcA* and those involved in the synthesis and deposition of the Hep layer of the heterocyst envelope; and late genes, those activated at 12–18 h, including many structural genes of the heterocyst-specific metabolic features such as those in the *cox2*, *cox3*, and *nif* operons. It should be noted that the induction of the early heterocyst differentiation genes overlaps that of genes involved in a general response to nitrogen stress including those for the scavenging of traces of combined nitrogen. Although these groups of genes can be distinguished in general terms, the specific time points that define them are necessarily coarse because the classification is based on information obtained in different laboratories generally using different growth settings (and thus nonidentical differentiation rates) for a given organism. In spite of this, valuable information on the sequence of gene activation during heterocyst differentiation has accumulated from time course northern or RT-PCR analysis of the expression of individual genes and by global studies. These studies have shown that many of the genes induced during the course of heterocyst differentiation are activated transiently, its expression returning to basal levels at a certain point thereafter. This is especially true for the genes induced at early or intermediate stages of the process, whose products may be required at a specific moment during differentiation. On the other hand, the study of the effects of the mutation of particular genes on the expression of others has provided valuable information on epistatic relationships.



By making use of transcriptional or translational fusions to reporter genes, mainly *gfp* (encoding the green fluorescent protein) but also *luxAB*, the spatial time course of activation along the filament has been studied for an increasing number of genes involved in heterocyst differentiation or function. The expression of some genes that are activated early upon combined-nitrogen stepdown is increased throughout the filament, although at somewhat higher levels in cells showing a spatial distribution reminiscent of further (pro)heterocysts. This is the case of, e.g. the *nrrA* gene encoding a response regulator with a role in heterocyst differentiation (Ehira & Ohmori, 2006a; Muro-Pastor, Olmedo-Verd, & Flores, 2006 see section 4.3 below). Unfortunately, no studies are available, to the best of our knowledge, for the spatial distribution of the induction of genes involved in the assimilation of traces of combined-nitrogen nutrients other than *amt1*, which is expressed at higher levels in vegetative cells than in heterocysts (Merino-Puerto *et al.*, 2010). On the other hand, genes for which a maximum induction takes place at intermediate stages of differentiation exhibit a more specific localization to (pro)heterocysts (e.g. the *ntcA* gene (Olmedo-Verd, Muro-Pastor, Flores, & Herrero, 2006)). It should be taken into account that the relative high stability of the GFP usually precludes visualization by this approach of the drop in gene expression for transiently expressed genes as it can be observed detecting mRNA.

### 3.3. Genes Active in the Mature Heterocyst

Because of the transient induction of many genes expressed at early to intermediate times during differentiation, the transcriptional pattern prevailing during a significant part of the differentiation process, which likely takes place under a condition of nitrogen stress, differs from that taking place in the mature heterocyst. For a number of genes activated late during heterocyst differentiation, induction has been shown to exhibit a tight spatial specificity, expression remaining high after completion of differentiation. This is the case of the *pipX* gene, encoding a regulatory factor that supports late gene expression in the differentiating cells, whose spatio-temporal specificity has been studied (Valladares *et al.*, 2011). This gene is activated upon nitrogen stepdown with a burst at c. 9–12 h, which is maintained until differentiation is completed (c. 24 h). Also, some genes involved in heterocyst function have been shown to be activated at medium to late stages of differentiation and to remain highly expressed in the mature heterocyst. This is the case of, e.g. the *nifHDK* operon encoding nitrogenase (Elhai & Wolk, 1990), the *ald* gene encoding a heterocyst-specific alanine



dehydrogenase (Pernil et al., 2010) and *invB* encoding a heterocyst-specific invertase (López-Igual et al., 2010).

### 3.4. Genes Repressed during Differentiation

The expression of many genes has been shown to decrease in *Anabaena* sp. strain PCC 7120 during a time course after combined nitrogen deprivation. Among these, transcripts of genes that encode phycobiliproteins are conspicuous. These and the transcripts of genes related to photosystem I, photosystem II, chlorophyll antenna, photosynthetic electron transport and ATP synthesis drop in abundance early but return to the initial levels at 24 h (Ehira & Ohmori, 2006a; see section 3.5 below). These decreases detected at the whole filament level should reflect a phenomenon that takes place throughout the filament. In contrast, transcripts of genes of the Calvin cycle and gluconeogenesis decreased to about half of the initial levels by 8 h after nitrogen deprivation. In the case of *rbcL* (encoding a subunit of Rubisco; Curatti, Giarrocco, & Salerno, 2006; Elhai & Wolk, 1990) and *susA* (encoding the sucrose cleavage enzyme sucrose synthase; Curatti et al., 2006), it has been shown, by making use of transcriptional fusions, that repression takes place specifically in (pro)heterocysts. Apart from metabolism genes, remarkable cases of genes repressed in heterocysts are those of *glnB* encoding the P<sub>II</sub> protein (Paz-Yepes et al., 2009), whose repression may be related to the need of activity of PipX (Valladares et al., 2011), for which the P<sub>II</sub> has been proposed to be an antagonist in the unicellular cyanobacterium *Synechococcus elongatus* (Llácer et al., 2010), and *fisZ* (Wang & Xu, 2005), whose repression may be related to the nondividing, differentiation-at-terminus character of the heterocyst.

### 3.5. Global Studies of Gene Expression

Several global transcriptional analyses of the responses of heterocyst-forming cyanobacteria to combined nitrogen deprivation have been published recently. Two main types of analysis have been performed, one based on DNA microarrays and the other based on deep-sequencing technology. In addition to the technique used to detect the transcription levels, there are some differences between the different analyses concerning the incubation conditions of the cyanobacteria. The microarray analysis of Ehira & Ohmori (2006a) was performed using filaments of *Anabaena* sp. strain PCC 7120 and an *nrrA* mutant (see section 4.3 below) grown with nitrate as the nitrogen source and transferred to a medium lacking combined nitrogen (liquid cultures bubbled with

CO<sub>2</sub>-enriched air). Samples were taken at time 0 and 3, 8 and 24 h after nitrate withdrawal. The deep-sequencing analysis carried out by Flaherty *et al.* (2011) was performed using filaments of *Anabaena* sp. strain PCC 7120 grown in an ammonium-containing medium and transferred to a medium lacking combined nitrogen (liquid cultures) for 6, 12 and 21 h. The results from these two analyses differ specially at the first time points since the microarray analysis started from filaments grown in the presence of nitrate, which, in regulatory terms, is a less-stringent nitrogen source compared to ammonium. Indeed, the master regulator of nitrogen assimilation genes, NtcA, is active under these conditions, and activates the expression of genes involved in the assimilation of nitrate and nitrite such as those in the *nirA* operon (Frías, Flores, & Herrero, 1994).

### 3.5.1. Microarray analysis in *Anabaena* sp. strain PCC 7120

The microarray analysis of Ehira & Ohmori (2006a), as re-evaluated by Xu *et al.* (2008), showed that the overall abundance of mRNA in wild-type *Anabaena* decreased after nitrogen stepdown. This can be explained by a generalized drop in transcription of the genes most highly expressed under nitrogen-replete conditions, remarkably those encoding the phycobiliproteins. Nonetheless, the authors detected 495 genes that increased and 196 that decreased specifically in expression at some point upon combined-nitrogen deprivation. They found that 51% of the genes whose expression increase at 3 h also do so at 8 h, and 71% of the genes whose expression decrease at 3 h also decrease at 8 h, which indicates a considerable overlapping in the transcriptional patterns operating during these stages of differentiation. In contrast, only 2% of the genes induced at 3 h remain so at 24 h and only 7% of those induced at 8 h remain so at 24 h.

Some genes encoding proteins involved in the assimilation of urea (the ABC-type uptake transporter UrtABCDE) and scavenging of ammonium (Amt translocators) are induced early upon nitrate withdrawal. The expression of genes in the *cph1* cluster for cyanophycin metabolism increases significantly at 3 h and remains high at 8 h. However, by 24 h, the levels of expression of genes involved in cyanophycin metabolism, as well as those of genes involved in urea and ammonium transport, were similar to the initial levels. In contrast, at 24 h, the *nifH*, *nifB* and *nifE* gene clusters are conspicuously induced. Regarding carbon metabolism, by 8 h expression of the *rbcLS* genes specific of the Calvin cycle decreased, whereas those specific for the oxidative pentose phosphate pathway increased. By 24 h, transcription

of the Calvin cycle genes returned to the initial levels while transcription of the oxidative pentose phosphate pathway genes remained high, which is consistent with known metabolic aspects of vegetative cells and heterocysts, respectively.

### **3.5.2. Directional RNA sequencing in *Anabaena* sp. strain PCC 7120**

The study by [Flaherty et al. \(2011\)](#) was performed using directional RNA-Seq to analyse the *Anabaena* transcriptome during nitrogen stepdown. By using this technique, they obtained information on transcript abundance and boundaries, including detection of operons, and on the length of the untranslated region (UTR) of each transcript. The results, represented in RPKM (reads per kilobase of coding sequence model per million mapped reads in the sample), showed that most of the genes differentially transcribed upon nitrogen deprivation were activated. The fact that genes known to be regulated early after nitrogen deprivation, such as *amt4* and *amt1* ([Paz-Yepes, Merino-Puerto, Herrero, & Flores, 2008](#)) or the *urt* operon ([Valladares, Montesinos, Herrero, & Flores, 2002](#)), were not yet differentially expressed at 6 h in this analysis suggests that the acclimation to nitrogen-depleted medium in this work was delayed with respect to previous analyses (hence, we have chosen not to include this time point in [Table 8.1](#)). On the chromosome, 434 genes were substantially upregulated at 12 h and 396 genes were upregulated at 21 h. In contrast, only 32 and 35 genes were downregulated at 12 h and 21 h, respectively. Among the genes showing the highest induction 12 h after nitrogen stepdown, there were those in the Hep island, a number encoding glycosyl transferases (probably involved in the formation of the heterocyst envelope too), and several encoding regulators (including NtcA and PatS). The genes activated at both 12 and 21 h included those encoding some enzymes expressed in the mature heterocyst, such as *ald* ([Pernil et al., 2010](#)), *cphB1* ([Picossi et al., 2004](#)), *glnA* ([Valladares et al., 2004](#)), and *invB* ([López-Igual et al., 2010](#)). The genes showing highest induction at 21 h were involved in the function of the heterocyst, including the *nifHDK* gene cluster, the *cox3* operon, *hupLS* (encoding an uptake hydrogenase), etc., and in the formation of the Hgl layer.

### **3.5.3. Transcription start point mapping in *Anabaena* sp. strain PCC 7120**

The transcriptomic analysis carried out by [Mitschke, Vioque, Haas, Hess, and Muro-Pastor \(2011\)](#) addressed the differential use of transcriptional start points (TSPs) in ammonium-grown cells and in cells incubated for 8 h in

**Table 8.1** Comparison of the expression after nitrogen stepdown of some genes likely involved in heterocyst differentiation. Data taken from global studies carried out with *Anabaena* sp. strain PCC 7120 and *Nostoc punctiforme*. The values are expressed as fold induction at the indicated hours after combined-nitrogen withdrawal

<i>Anabaena</i> ORF      Function		Xu et al., 2008 (after Ehira & Ohmori 2006a)			Flaherty et al., 2011	
		3 h	8 h	24 h	12 h	21 h
<i>all0050</i>	Unknown protein	1.73	2.09	3.69	24.4	42.0
<i>all0283</i>	WD-40 repeat protein	1.18	0.69	2.79	2.52	4.24
<i>all0438</i>	Ser/Thr kinase with WD-40 repeat	6.37	14.2	—	22.9	9.04
<i>all0521</i>	2-component RR. PatA	1.32	0.58	0.82	1.62	1.39
<i>all0935</i>	Hypothetical protein	1.10	2.92	1.35	3.80	2.35
<i>all1214</i>	Undecaprenol kinase	5.75	15.3	2.55	7.86	3.67
<i>all1523</i>	Regulatory protein	9.99	3.30	3.55	0.657	1.21
<i>all1692</i>	sigma factor; SigC	3.82	5.34	1.98	11.8	5.06
<i>all1731</i>	Protein phosphatase PrpJ	2.34	2.22	1.50	2.97	1.35
<i>all1747</i>	Unknown protein	1.59	2.65	1.39	3.22	1.58
<i>all2038</i>	Unknown protein	2.06	15.4	2.23	29.8	9.40
<i>all2059</i>	Unknown protein	1.50	6.11	2.26	1.45	0.596
<i>all2128</i>	Unknown protein	1.37	1.96	1.25	36.7	79.4
<i>all2342</i>	Phage shock protein A	1.17	0.99	0.77	1.43	1.33
<i>all2571</i>	Transport associated	2.03	13.4	9.97	26.6	14.7
<i>all2736</i>	OM hypothetical protein	0.98	2.26	0.85	2.64	1.61
<i>all2760</i>	Ser/Thr kinase	2.10	8.46	1.84	7.51	2.39
<i>all2965</i>	Unknown protein	1.80	—	8.00	25.6	43.8
<i>all3580</i>	Unknown protein	2.68	1.78	2.24	3.28	3.22
<i>all3660</i>	2-component RR	1.43	1.31	1.90	3.70	2.28
<i>all3788</i>	2-component RR	—	3.31	0.54	4.26	1.52
<i>all3792</i>	Hypothetical protein	1.33	—	0.08	6.19	2.69
<i>all4220</i>	Hypothetical protein	1.30	3.17	1.54	0.852	0.660
<i>all4246</i>	K <sup>+</sup> -dependent ATPase	1.00	2.83	1.79	2.33	2.49
<i>all4822</i>	Similar to $\beta$ -lactamase	0.57	4.09	1.95	27.9	13.8
<i>all4962</i>	Unknown protein	1.66	3.97	1.14	9.69	5.63
<i>all5263</i>	Sigma factor SigA	1.10	0.75	0.64	2.01	2.13
<i>alr0181</i>	Hypothetical protein	1.07	1.51	1.07	2.38	2.87
<i>alr0518</i>	Hypothetical protein	0.24	2.68	0.60	1.93	0.619
<i>alr0566</i>	Unknown protein	1.63	1.74	1.33	3.92	4.34
<i>alr0627</i>	Glycoside hydrolase	0.37	0.98	0.35	1.51	2.60

Mitschke et al., 2011			Christman et al., 2011						
TSP*	8 h	<i>Nostoc</i> ORF	0.5 h	1 h	3 h	6 h	12 h	18 h	24 h
54960	99.1	NO <sup>§</sup>							
316513	87.0	NO							
518833	510	NpR1546	0.74	0.80	1.02	1.39	2.00	2.06	1.52
518845	32.2								
614365 <sup>‡</sup>	41.0	NpF5682	NDT <sup>  </sup>						
1088088	24.4	NpF5956	NDT						
1431985	28.0	NO							
1785637 <sup>‡</sup>	18.0	NpR4194	1.51	1.33	0.97	0.86	1.01	0.89	0.95
2022661 <sup>‡</sup>	30.0	NpF0996	2.27	1.77	0.95	0.81	0.91	0.44	0.80
2084891 <sup>‡</sup>	61.0	NpF1519	NDT						
2101843	715	NpR1311	1.29	1.32	1.42	1.54	1.58	1.37	1.00
2439485	173	NpR2952	0.74	0.97	2.28	5.28	8.00	4.44	2.08 <sup>¶</sup>
2467917	45.7	NpF4230	NDT						
2552918	55.4	NO							
2825529	31.0	NpR3963	NDT						
3072950	113	NpR5742	1.33	1.32	1.61	3.34	10.6	5.94	1.87 <sup>¶</sup>
3333817	30.5	NpF1721	NDT						
3357699	103	NpR6098	NDT						
3607611	82.7	NO							
4326127	119	NO							
4418454 <sup>‡</sup>	104	NpF0832	1.42	1.47	1.67	1.91	2.07	1.74	1.14
4579255	30.0	NpR4165	NDT						
4583904	19.4	NpR4169	1.01	1.03	1.12	1.23	1.39	1.41	1.28 <sup>¶</sup>
5056900	176	NpF6107	NDT						
5092719	162	NpR0302	1.41	1.34	1.15	1.08	1.39	1.93	1.73 <sup>¶</sup>
5742628	19.0	NpF4556	NDT						
5921933	142	NpR0895	1.09	1.13	1.29	1.52	1.80	1.79	1.46
6279248 <sup>‡</sup>	36.5	NpF6374	1.61	1.60	1.56	1.48	1.37	1.25	1.14
6279444 <sup>‡</sup>	26.5								
191769	54.0	NpR1427	NDT						
610579	109	NpR1875	NDT						
660489	62.8	NO							
727113 <sup>‡</sup>	11.7	NpR6304	0.74	0.86	1.25	1.35	1.07	1.58	2.14 <sup>¶</sup>

Continued

**Table 8.1** Comparison of the expression after nitrogen stepdown of some genes likely involved in heterocyst differentiation. Data taken from global studies carried out with *Anabaena* sp. strain PCC 7120 and *Nostoc punctiforme*. The values are expressed as fold induction at the indicated hours after combined-nitrogen withdrawal—cont'd

<i>Anabaena</i> ORF      Function		Xu et al., 2008 (after Ehira & Ohmori 2006a)			Flaherty et al., 2011	
		3 h	8 h	24 h	12 h	21 h
<i>alr0717</i>	Hypothetical protein	1.72	1.85	1.41	5.12	6.25
<i>alr0819</i>	Alkaline invertase	2.37	2.92	0.93	3.35	2.21
<i>alr1112</i>	Probable transglycosylase	1.71	4.33	1.11	5.89	2.28
<i>alr1238</i>	Clp protease	1.75	1.80	1.41	1.47	1.44
<i>alr1302</i>	Acetyltransferase	0.65	0.70	1.09	2.04	1.19
<i>alr1677</i>	Hypothetical protein	2.20	7.27	1.86	14.8	10.1
<i>alr2076</i>	Hypothetical protein	5.66	4.82	0.88	1.44	1.11
<i>alr2339</i>	HetR	1.51	2.32	1.06	4.36	3.75
<i>alr2478</i>	Hypothetical protein	2.84	7.90	1.62	12.3	5.77
<i>alr2514</i>	CoxB2	1.41	85.6	10.3	41.4	76.8
<i>alr2717</i>	Hypothetical protein	1.08	1.11	1.03	3.20	2.62
<i>alr2790</i>	Unknown protein	1.85	10.1	1.39	9.77	8.03
<i>alr2818</i>	HetP	1.66	139	6.61	10.1	5.63
<i>alr2822</i>	Von Willebrand factor, type A	3.73	46.4	6.67	263	104
<i>alr2826</i>	Hypothetical protein	3.39	205	10.8	430	190
<i>alr2830</i>	RfbC	1.70	142	4.38	263	90.1
<i>alr2832</i>	Glycosyltransferase	2.98	–	71.6	3.4·10 <sup>38</sup>	3.4·10 <sup>38</sup>
<i>alr2833</i>	Hypothetical protein	2.81	170	–	740	227
<i>alr2834</i>	HepC	–	–	8.00	47.8	19.3
<i>alr2835</i>	HepA	1.63		1.63	3.4·10 <sup>38</sup>	3.4·10 <sup>38</sup>
<i>alr2837</i>	Glycosyltransferase	7.23	252	3.04	3.4·10 <sup>38</sup>	3.4·10 <sup>38</sup>
<i>alr2933</i>	Transglycosylase A	1.66	3.91	3.91	24.4	7.24
<i>alr2947</i>	Unknown protein	0.46	0.61	0.64	4.06	4.67
<i>alr2948</i>	Putative zinc-binding oxidoreductase	1.27	0.82	0.63	4.07	4.91
<i>alr3096</i>	Phosphate permease	1.80	3.90	1.91	12.5	7.73
<i>alr3353</i>	Putative peptidase	1.18	1.12	0.56	3.63	2.38

Mitschke et al., 2011			Christman et al., 2011						
TSP*	8 h	<i>Nostoc</i> ORF	0.5 h	1 h	3 h	6 h	12 h	18 h	24 h
834297	26.5	NpF0167	1.74	1.68	1.48	1.26	1.00	0.90	0.93
942986 <sup>‡</sup>	56.5	NpF4643	1.17	1.19	1.27	1.41	1.73	2.13	2.62
1301890	19.0	NpR5647	NDT						
1301935	159								
1469842 <sup>‡</sup>	143	NpF6219	NDT						
1542635	17.0	NpF5731	NDT						
1999834 <sup>‡</sup>	61.0	NpF5744	0.99	1.04	1.29	1.68	2.33	2.46	1.99
2483108	25.7	NpR2710	1.09	1.04	1.16	2.16	5.86	3.01	1.73
2821366 <sup>‡</sup>	16.6	NpR1722	1.30	1.51	2.48	3.94	4.69	3.25	2.33 <sup>¶</sup>
2975615	20.5	NpR1537	NDT						
3021024	41.5	NpF0336	0.98	0.95	1.01	1.64	8.00	24.25	9.13 <sup>¶</sup>
3311698	81.0	NpF0339	1.01	0.99	0.99	1.16	2.20	3.66	2.69
3391021	114	NO							
3431807 <sup>‡</sup>	71.0	NpR1542	1.13	1.25	1.80	2.77	4.00	3.07	1.25
3436144 <sup>‡</sup>	78.0	NpR1083	0.90	0.98	1.36	1.99	3.01	2.79	1.59
3436409 <sup>‡</sup>	60.2								
3436549	26.8								
3441875	36.5	NpR1079	0.89	1.00	1.84	4.96	9.58	2.27	1.88 <sup>¶</sup>
3441999	62.3								
3446301	74.5	NpR1074	0.91	1.03	1.89	4.86	11.2	4.14	1.80 <sup>¶</sup>
3448677	208	NpR1071	0.81	1.06	2.85	9.25	34.1	30.06	6.45 <sup>¶</sup>
3449710	233	NpR1070	0.99	1.03	1.56	4.32	18.8	10.27	3.97 <sup>¶</sup>
3452463 <sup>‡</sup>	86.0	NpR1069	0.93	1.04	2.03	6.96	28.4	10.13	3.41 <sup>¶</sup>
3452765	286								
3453831 <sup>‡</sup>	94.0	NpR1068	1.04	1.02	1.23	2.55	6.96	3.01	1.67 <sup>¶</sup>
3457231	15.4	NpR1066	0.86	1.00	1.69	3.14	5.86	4.79	1.73 <sup>¶</sup>
3569154	14.9	NpR5306	NDT						
3585345	68.4	NO							
3585966 <sup>‡</sup>	57.5	NpR1898	NDT						
3585972	115								
3585991	35.0								
3747939	14.3	NpR1901	NDT						
4056311 <sup>‡</sup>	22.7	NpF6078	NDT						

Continued

**Table 8.1** Comparison of the expression after nitrogen stepdown of some genes likely involved in heterocyst differentiation. Data taken from global studies carried out with *Anabaena* sp. strain PCC 7120 and *Nostoc punctiforme*. The values are expressed as fold induction at the indicated hours after combined-nitrogen withdrawal—cont'd

<i>Anabaena</i> ORF      Function		Xu et al., 2008 (after Ehira & Ohmori 2006a)			Flaherty et al., 2011	
		3 h	8 h	24 h	12 h	21 h
<i>alr3361</i>	Hypothetical protein	1.63	2.44	0.86	3.04	3.05
<i>alr3431</i>	Copper amine oxidase	1.18	–	2.95	5.88	3.15
<i>alr3698</i>	HepB	2.00	27.6	4.02	3.4·10 <sup>38</sup>	3.4·10 <sup>38</sup>
<i>alr3710</i>	DevB	2.92	45.4	–	17.8	8.78
<i>alr3808</i>	Nutrient-stress induced DNA-binding protein	2.48	10.8	2.02	3.51	2.41
<i>alr3817</i>	Unknown protein	7.22	8.48	1.61	3.72	2.79
<i>alr3952</i>	DevH	1.04	3.93	1.22	4.61	3.01
<i>alr4077</i>	Hypothetical protein	1.50	1.50	0.91	1.73	1.64
<i>alr4329</i>	Anti-anti sigma factor	7.08	9.02	2.36	6.88	2.35
<i>alr4392</i>	NtcA	1.21	5.92	1.43	5.55	2.47
<i>alr4485</i>	ABC-type permease	0.91	1.08	1.30	1.67	1.69
<i>alr4984</i>	Unknown protein	1.63	71.0	8.06	25.4	5.66
<i>alr5251</i>	2-component RR	1.79	26.3	1.26	3.85	3.37
<i>asl1305</i>	Unknown protein	1.79	5.41	4.37	5.91	2.18
<i>asl1778</i>	Unknown protein	3.27	7.04	4.12	18.9	11.8
<i>asr0485</i>	Regulatory factor PipX	1.23	4.87	2.48	5.50	7.39
<i>asr1277</i>	PS II protein PsbI	0.86	1.12	1.01	1.38	0.972
<i>asr1734</i>	Unknown protein	8.29	14.8	4.60	24.6	8.12
<i>asr1775</i>	Unknown protein	0.93	1.52	2.88	1.95	2.16

\*TSP: Transcriptional start point showing increased use in the wild type but not in the *hetR* mutant (Mitschke et al., 2011). These TSPs were located within the upstream 200 nucleotides of the assigned ORF except when indicated otherwise. The TSPs corresponding to ORFs encoding unknown or hypothetical proteins with RPKM <3 (Flaherty et al., 2011) are not shown in the table.

†TSPs further upstream than 200 nucleotides of the assigned ORF, with no apparent ORF between the TSP and the assigned ORF.



Mitschke et al., 2011			Christman et al., 2011						
TSP*	8 h	<i>Nostoc</i> ORF	0.5 h	1 h	3 h	6 h	12 h	18 h	24 h
4066829 <sup>†</sup>	119	NpF3994	NDT						
4137698	66.0	NpR4934	1.18	1.28	1.69	2.33	3.12	2.62	1.39
4465653	113	NpF2953	0.94	1.01	1.27	1.68	2.23	2.07	1.34 <sup>‡</sup>
4478416 <sup>‡</sup>	509	NpR5576	1.01	0.98	1.06	1.91	12.3	39.40	8.63 <sup>‡</sup>
4601709 <sup>‡</sup>	22.9	NpR5799	0.94	1.03	1.41	2.04	2.83	2.30	1.09 <sup>‡</sup>
4601949	47.0								
4616158	46.0	NO							
4769713	12.4	NpR6193	0.84	0.94	1.40	2.06	2.38	1.75	1.25
4907285 <sup>†</sup>	241	NpF5965	1.01	1.04	1.16	1.31	1.53	1.52	1.30
5180314	25.7	NpR3892	1.10	1.16	1.39	1.73	2.10	1.87	1.22
5265235 <sup>‡</sup>	109	NpF5511	0.93	1.00	1.36	1.96	3.01	3.12	2.14 <sup>‡</sup>
5369311	14.2	NO							
5953754 <sup>†</sup>	58.0	NpR1877	NDT						
5954131	235								
6265857	13.2	NpR4435	1.04	1.01	0.93	0.83	0.71	0.67	0.69
1547808	26.7	NO							
2137186	21.3	NpF2362	NDT						
2137192	29.5								
580341 <sup>‡</sup>	21.2	NpR1700	1.05	0.99	0.95	1.31	2.66	2.11	1.40
580704 <sup>‡</sup>	83.8								
1516291	119	NO							
2086181 <sup>†</sup>	57.2	NpR1517	1.56	1.60	1.78	1.97	2.00	1.58	0.97
2134180	23.4	NpR6320	NDT						

<sup>†</sup>TSPs further upstream than 200 nucleotides of the assigned ORF validated experimentally (see Mitschke et al., 2011 and references therein; Young-Robbins, Risser, Moran, Haselkorn, & Callahan, 2010).

<sup>‡</sup>NO: No orthologue found.

‡NDT: Not differentially transcribed.

\*These genes were also upregulated in steady-state cultures grown in the absence of combined nitrogen.

the absence of combined nitrogen (bubbled cultures enriched with CO<sub>2</sub>) both in the wild-type strain and in a *hetR* mutant that is unable to differentiate heterocysts (Buikema & Haselkorn, 1991). They identified >900 putative TSPs whose use increased in response to nitrogen deficiency, of which 209 were not induced in the *hetR* mutant suggesting that they are involved in heterocyst differentiation. The TSPs that were independent of HetR include some corresponding to genes involved in the assimilation of sources of nitrogen alternative to ammonium and in scavenging of traces of ammonium, as well as of genes involved in the general response to nitrogen deprivation. Indeed, as previously described for a number of experimentally analysed genes (e.g. Frías *et al.*, 1994; Luque, Flores, & Herrero, 1994; see also Herrero, Muro-Pastor, Valladares, & Flores, 2004; and section 4.1 below), a significant proportion of TSPs in this group are preceded by sequences matching the canonical (Class II) NtcA-activated promoter of cyanobacteria. Mitschke *et al.* (2011) also identified 28 TSPs whose use was repressed in response to nitrogen limitation. Although these authors define the NtcA regulon by the TSPs showing transcriptional changes common to the wild type and the *hetR* mutant, it should be noted that a number of *hetR*-regulated promoters of heterocyst differentiation genes have been experimentally demonstrated to be regulated by NtcA in a direct manner (see, e.g. Camargo, Valladares, Flores, & Herrero, 2012; Olmedo-Verd, Valladares, Flores, Herrero, & Muro-Pastor, 2008). Thus, the NtcA regulon includes also promoters that *in vivo* respond to HetR.

The two studies based on deep sequencing performed by Flaherty *et al.* (2011) and Mitschke *et al.* (2011) have shown the presence of multiple non-coding RNA transcripts, some of which exhibit nitrogen-dependent regulation and, in some cases, HetR-dependent regulation, as well as multiple antisense transcripts, suggesting antisense transcription to 39% of all genes in the genome of *Anabaena* sp. strain PCC 7120. Antisense transcription and noncoding RNAs perhaps underlie important mechanisms of regulation during the response to combined-nitrogen deprivation.

#### **3.5.4. Microarray analysis in *Nostoc punctiforme***

Two DNA microarray-based transcriptomic analyses of the response to nitrogen limitation have been performed using *Nostoc punctiforme* (Campbell *et al.*, 2007; Christman, Campbell, & Meeks, 2011). In addition to producing heterocysts, the vegetative cells of this cyanobacterium can differentiate into hormogonial cells and akinetes. Campbell *et al.* (2007) focused on the comparison of steady-state cultures of a spontaneous hormogonium-deficient

mutant growing with ammonium as the nitrogen source or without combined nitrogen. They identified 495 genes that were differentially expressed in the  $N_2$ -grown cells, most of which (373) were upregulated. Christman et al. (2011), on the other hand, described a transcriptional analysis of the changes produced after nitrogen stepdown in filaments undertaking the process of heterocyst differentiation. They used the spontaneous hormogonium-deficient mutant grown with ammonium as the nitrogen source and incubated in a nitrogen-depleted medium for 0.5, 1, 3, 6, 12, 18, and 24 h (shaken liquid cultures). A total of 1036 genes were significantly up- or down-regulated along the 24-h induction. The authors presented two classifications of the regulated genes: the first one grouped the genes by functionality, with 18% of the genes belonging to adaptive metabolism and 32% to core metabolism, and the second grouped the genes according to their temporal pattern of expression, defining six clusters of genes. Cluster 4 includes genes that are highly activated at late time points, such as the *hgl* and *nif* genes. Cluster 6 includes the genes with the highest expression at 12 h upon induction, such as the *hep* genes. Overall, the timeframes at which these genes are induced are the same as in *Anabaena* sp. strain PCC 7120 (Ehira & Ohmori, 2006a; Flaherty et al., 2011; Xu et al., 2008).

Campbell, Christman, and Meeks (2008) have also compared the results of the transcriptomic analysis during heterocyst differentiation in the hormogonium-deficient mutant described above with the results of a similar transcriptomic analysis carried out in wild-type filaments during hormogonia differentiation in response to nitrogen deprivation. They conclude that the hormogonium-differentiation program is much more complex than the heterocyst differentiation program, although there are some common differentially expressed genes. The expression of *nrrA*, for example, was activated at 0.5 h of nitrogen deprivation in both processes and remained elevated throughout development, although the induction was more discrete during hormogonia differentiation. However, other regulatory genes induced during heterocyst differentiation, such as *hetR* or *ntcA*, were not induced during hormogonia differentiation, which suggests a general role for *nrrA* in the response to nitrogen stress rather than a specific role in heterocyst differentiation (see section 4.3 below).

Christman et al. (2011) also compared the genes differentially expressed at 24 h after nitrogen stepdown to the genes expressed differentially in diazotrophic steady-state cultures (Campbell et al., 2007). They found more genes differentially expressed at 24 h than in steady-stated cultures, most of which were upregulated (559 vs. 378 genes upregulated and 378 vs.

123 genes downregulated at 24 h and steady-state cultures, respectively). The majority of the 231 genes that were upregulated under both physiological conditions encode known heterocyst regulatory and function proteins. The set of 328 genes upregulated at 24 h but not differentially expressed in steady-state cultures include genes involved in transcriptional regulation, cofactors, protein polymerization, transport and secondary metabolism, which would not be necessary once the cells have acclimated to the new growth condition.

In an attempt to compare some of the results of these global studies, we have summarized the transcriptional behaviour of some genes involved in heterocyst differentiation in both *Anabaena* sp. strain PCC 7120 and *Nostoc punctiforme*. Table 8.1 includes genes for which a HetR-dependent TSP (8 h) has been identified in strain PCC 7120 (Mitschke *et al.*, 2011). Together, the global studies described above suggest that 500–1000 genes change in expression during the period from the onset of combined nitrogen withdrawal to heterocyst performance. More genes (and regulated TSPs) increase than decrease in expression during acclimation to combined-nitrogen deprivation. Most early-induced genes are expressed transiently, whereas many medium- to late-induced genes remain active once heterocyst differentiation has been completed, although a fraction of them return to basal levels of expression in steady-state diazotrophic growth.



## 4. MECHANISMS OF GENE REGULATION DURING HETEROCYST DIFFERENTIATION

### 4.1. The NtcA and HetR Regulators

Gene regulation during heterocyst differentiation is orchestrated by the combined action of two principal regulators, the global transcriptional regulator NtcA that activates, and in some cases represses, genes as a function of the carbon-to-nitrogen balance of the cells, and the differentiation-specific factor HetR. Mutants lacking either of these regulators do not show any sign of heterocyst differentiation upon nitrogen deprivation (Frías *et al.*, 1994; Ramasubramanian, Wei, & Golden, 1994). In addition, when studied at the whole filament level by northern or primer extension analyses, the increase in gene expression that in the wild-type strain is observed upon nitrogen stepdown for genes involved in heterocyst differentiation does not take place in *ntcA* mutants. Mutation of *hetR* also impairs the expression of many genes activated during heterocyst differentiation, although for some of them the effect is small.

NtcA is a protein of c. 220–242 amino acids present and highly conserved in all cyanobacteria so far analysed that is similar to proteins of the CRP/FNR family of transcriptional regulators (Herrero, Muro-Pastor, & Flores, 2001; Luque & Forchhammer, 2008). NtcA binds DNA as a dimer at sites with the consensus sequence GTAN<sub>8</sub>TAC, which in activator sites is frequently centred at c. 41.5 nucleotides upstream from the TSP of the regulated gene. NtcA sites in this position are accompanied by a –10 promoter determinant with the consensus sequence TAN<sub>3</sub>T, conforming the so-called canonical NtcA-activated promoter (Herrero et al., 2001; Luque et al., 1994), which matches the structure of the bacterial Class II activator-dependent promoters (Busby & Ebright, 1999). In some other NtcA-activated promoters, some of which represent Class I promoters, a single site for NtcA binding is found centred further upstream from the –41.5 position (see Busby & Ebright, 1999; Luque & Forchhammer, 2008), whereas in others more than one NtcA-binding site are present and/or additional regulatory factors may participate (see sections 4.2 and 4.3 below). In the case of repression by NtcA, the binding site for this regulator is centred downstream of the –41.5 position and could overlap the –10 determinant of the promoter or be located within the gene (see Herrero et al., 2001; Luque & Forchhammer, 2008; Olmedo-Verd et al., 2008). Repression by NtcA could be responsible for the exclusive expression in vegetative cells, or preferential expression in vegetative cells relative to heterocysts, of genes such as *rbcLS* (Ramasubramanian et al., 1994), *hanA* (encoding a histone-like HU protein; Khudyakov & Wolk, 1996) or *gor* (encoding glutathione reductase; Jiang, Hellman, Sroga, Bergman, & Mannervik, 1995).

NtcA can specifically bind DNA in the absence of effectors (Luque et al., 1994). However, 2-oxoglutarate has been shown to increase NtcA binding to a number of activated promoters of different cyanobacteria (see, e.g. Olmedo-Verd et al., 2008; Vázquez-Bermúdez, Herrero, & Flores, 2002a). This has been recently confirmed with the resolution of the crystal structure of NtcA from *Anabaena* sp. strain PCC 7120 (Zhao et al., 2010) and the unicellular cyanobacterium *Synechococcus elongatus* (Llácer et al., 2010), which closely resembles that of the CRP protein from *E. coli*. In the case of *Anabaena*, comparison of the structure of the dimeric apoprotein to that in complex with 2-oxoglutarate showed that this effector produces a change in the orientation of the two DNA-recognition helices of the NtcA dimer, which nonetheless could contact DNA in the absence of the effector, with the effect of enhancing its DNA-binding activity. This is in contrast to the

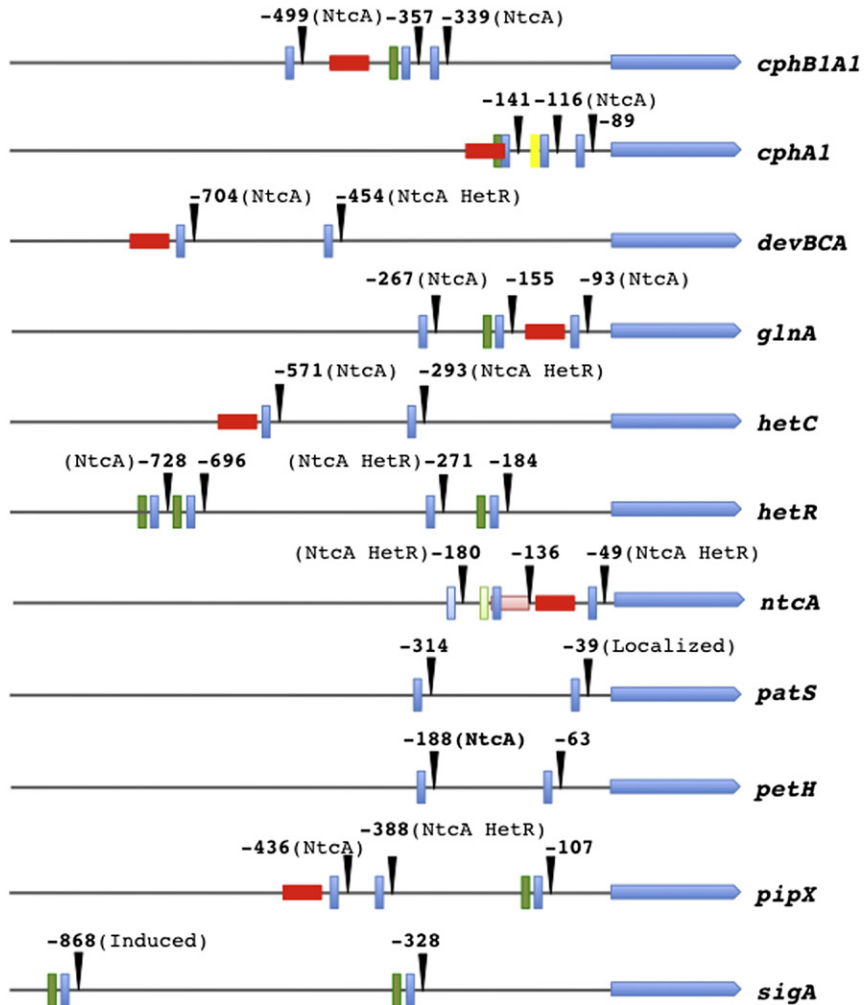
transition from the off- to the on-state induced by cAMP binding in CRP (Popovych, Tzeng, Tonelli, Ebright, & Kalodimos, 2009).

HetR is a c. 299-amino-acid protein for which structural motifs could be identified only after determination of the crystal structure of the protein from the thermotolerant cyanobacterium *Fischerella* MV11 (Kim *et al.*, 2011). This protein is a dimer with a central core made of the C- and the N-terminal regions of the two subunits, the latter conforming a DNA-binding unit made of two helix–turn–helix motifs, and two protruding globular flaps that could provide for heterologous contacts. HetR has been shown to bind in vitro DNA fragments upstream of several genes involved in heterocyst differentiation: *hetR*, *hepA*, and *patS* (Huang, Dong, & Zhao, 2004; Risser & Callahan, 2007), *hetP* (Higa & Callahan, 2010), *pknE* (Saha & Golden, 2011) and *hetZ* (Du, Cai, Hou, & Xu, 2012). In the case of *hetP*, a 17-bp inverted repeat sequence in DNA has been described as the HetR-binding sequence (Higa & Callahan, 2010). The features of this target are in agreement with those of the HetR DNA-binding domain (Kim *et al.*, 2011). However, the HetR-binding sequence found in *hetP* is absent from the DNA fragments of other promoters to which HetR has been described to bind, although the sequence upstream of *hetZ* is related to that of *hetP*. Thus, although the flap motifs of HetR could provide additional contacts for binding to less-suited DNA sequences (Kim *et al.*, 2011), in most cases the DNA sequences determining HetR affinity are unsolved, as is also the mechanism for HetR-dependent activation of gene expression.

## 4.2. Complex Promoter Regions in Heterocyst Genes

Many genes involved in heterocyst differentiation that have been experimentally characterized are preceded by complex promoter regions integrated by several consecutive promoters including NtcA-activated promoters, vegetative-type promoters and promoters that, being positively affected by NtcA and HetR, do not show a recognizable promoter structure. Figure 8.2 shows a schematic representation of the complex promoter regions of a number of such genes, which are described in Table 8.2.

In a few cases, the spatiotemporal specificity of the expression and the mechanism for transcription activation has been studied for individual promoter components of a complex promoter region. At Class II NtcA-activated promoters of the *devBCA* operon and the *hetC* and *nrrA* genes (the latter expressed from a single TSP), NtcA together with 2-oxoglutarate increase approximately twofold binding of RNA polymerase (RNAP) to the promoter DNA in vitro and are stringently required for the formation of the



**Figure 8.2** Complex promoter regions of some *Anabaena* sp. strain PCC 7120 genes participating in heterocyst differentiation or function (Table 8.2). The experimentally determined dependence on NtcA and HetR is indicated. Black triangles indicate TSPs; blue boxes, -10 determinants; green boxes, -35 determinants; yellow box, a putative UP element; red boxes, NtcA-binding sites. Imperfect determinants are indicated with the corresponding light colours. See the colour plate.

transcriptionally active open promoter complexes (Valladares, Flores, & Herrero, 2008). For the cases of *devBCA* (Camargo et al., 2012) and *ntca* (Ehira & Ohmori, 2006a), induction upon nitrogen stepdown has been shown, by making use of transcriptional fusions to the *gfp* gene, to take place in all

**Table 8.2** Consecutive promoters in some *Anabaena* sp. strain PCC 7120 genes involved in heterocyst differentiation and function

Gene(s)	Comments	Reference(s)
<i>phb1A1</i>	Operon expressed from three consecutive promoters. The one generating TSP -357 is a vegetative-type promoter, and those generating TSPs -339 (Class I NtcA-dependent, being the main promoter used in heterocysts) and -499 are inducible and directly regulated by NtcA. In addition, <i>phb1A1</i> is expressed monocistronically from two vegetative-type promoters (TSP -89, preceded by a -10 box and a UP element, and TSP -141 preceded by -10 and -35 determinants) and a Class II NtcA-activated promoter (TSP -116), which is active in heterocysts.	Picossi et al., 2004
<i>devBCA</i>	Operon expressed from a proximal promoter (TSP -454) with a recognizable -10 box, dependent on NtcA and HetR, directly activated by NtcA and induced in proheterocysts, and a distal, Class II NtcA-activated promoter (TSP -704) induced in vegetative cells and, at higher levels, in proheterocysts.	Fiedler, Muro-Pastor, Flores, & Maldener, 2001; Camargo et al., 2012
<i>glnA</i>	Gene expressed from a vegetative-type promoter (TSP -155 or -157) preceded by consensus -10 and -35 sequences, and at least two inducible promoters, of which that producing TSP -93 represents a Class II NtcA-activated promoter and is the main promoter used in mature heterocysts, and that producing TSP -267 (or -275) depends on NtcA and is preceded by a putative -10 box.	Frías et al., 1994; Tumer et al., 1983; Valladares et al., 2004
<i>hetC</i>	Gene expressed from a proximal promoter (TSP -293) directing NtcA- and HetR-dependent increased expression localized to proheterocysts, which includes a putative -10 determinant, and a distal inducible promoter (TSP -571) representing a Class II NtcA-activated promoter.	Muro-Pastor, Valladares, Flores, & Herrero, 1999, 2009
<i>hetR</i>	Gene expressed from four consecutive promoters, a proximal one (TSP -184) active in the presence and absence of combined nitrogen and three inducible one producing TSP -271 that depends on NtcA and HetR and shows increased activity mainly in the differentiating cells; one producing TSP -696 that requires neither NtcA nor HetR; and one producing TSP -728 that shows a strong requirement for NtcA. The latter two show considerable induction both in vegetative cells and heterocysts. Putative -10 and -35 determinants can be recognized upstream from the -184, -696 and -728 positions, and a putative -10 box upstream of -271. NrrA has been described to bind at a sequence between -844 and -818.	Buikema & Haselkorn, 2001; Ehira & Ohmori, 2006b; Muro-Pastor et al., 2002; Rajagopalan & Callahan, 2010



<i>ntcA</i>	Gene expressed from three consecutive promoters: a proximal promoter (TSP -49) preceded by a putative -10 determinant and an NtcA-binding site centred at position -54.5 with regard to the TSP, which is activated at intermediate stages of heterocyst differentiation, is the major promoter used in mature heterocysts and, when isolated from other promoters, shows activity in all the cells of the filament, although expression increases in proheterocysts dependent on NtcA and HetR; a vegetative-type promoter (TSP -136) preceded by a putative -10 element and a poor -35 element, with an NtcA-binding site (centred at -143.5) that overlaps the -10 box and could have a repressor role on this promoter; and a distal promoter (TSP -180) activated transiently at intermediate stages of heterocyst differentiation, dependent on NtcA and HetR, upstream of which only an imperfect -10 box can be recognized.	Muro-Pastor et al., 2002; Olmedo-Verd et al., 2006, 2008; Ramasubramanian, Wei, Oldham, & Golden, 1996
<i>patA</i>	Gene expressed from a proximal promoter (TSP -305) that is activated upon N stepdown in all cells of the filament in a HetR-dependent manner and is negatively autoregulated, and from distal promoters (TSP -614 and TSP -645) whose combined expression increases early principally in the differentiating cells. Two putative NtcA-binding sites (centred at positions -110 and -249) have been implicated in <i>patA</i> activation. However, because of their location downstream from the reported TSPs, a direct activator role of NtcA bound to these sites on transcription from those TSPs is difficult to envision.	Bastet et al., 2010; Young-Robbins et al., 2010
<i>patS</i>	Gene transcribed from a weak promoter in vegetative cells (TSP -314) and a strong inducible one (TSP -39) in differentiating cells.	Yoon & Golden, 2001
<i>petH</i>	Gene expressed from a constitutive promoter (TSP -63) and an NtcA-dependent inducible promoter (TSP -188) that is functional in mature heterocysts. Both of them present recognizable -10 determinants.	Valladares, Muro-Pastor, Fillat, Herrero, & Flores, 1999
<i>pipX</i>	Gene expressed from three promoters: a Class II NtcA-activated promoter (TSP -436), a vegetative promoter (TSP -107) with consensus -10 and -35 boxes, and an NtcA- and HetR-dependent promoter (TSP -388) with a recognizable -10 box.	Valladares et al., 2011
<i>sigA</i>	Gene expressed from at least two promoters, of which one (TSP -328) is constitutive and another (TSP -868) is active with and without combined nitrogen, but its activity increases under N deprivation. Both promoters are preceded by good -10 and -35 hexamers.	Brahamsha & Haselkorn, 1991

cells of the filament, although at somewhat increased levels in differentiating cells. On the other hand, the HetR-regulated proximal promoters of *devBCA* (Camargo *et al.*, 2012) and *hetC* (Muro-Pastor, Flores, & Herrero, 2009), as well as that producing TSP -271 of *hetR* (Rajagopalan & Callahan, 2010), are activated in differentiating cells. In the case of the *devBCA* proximal promoter, in spite of the lack of a consensus NtcA-binding site, NtcA binds to DNA *in vitro* and, together with 2-oxoglutarate, directly activates transcription (Camargo *et al.*, 2012), with HetR having some role in helping interaction of NtcA with degenerated NtcA-binding sites. Whether support of activation by NtcA is the only effect of HetR or whether, in other promoters, it can activate gene expression in the absence of NtcA should be clarified in the future.

In summary, Class II promoters would be activated throughout the filament early upon perception of nitrogen stress providing certain level of gene expression in all the cells, which for genes involved in heterocyst differentiation would be reinforced later in specific cells in response to a localized increase in NtcA levels. On the other hand, localized, and in some cases transient, activity of HetR-dependent promoters would reinforce the spatially localized gene expression. Thus, the contribution of the two promoter types to direct gene expression preferentially in the differentiating cells would respond to an amplification loop resulting from localized positive autoregulation of *ntcA* and *hetR*, both of which include mutually dependent NtcA- and HetR-dependent promoters (Buikema & Haselkorn, 2001; Muro-Pastor, Valladares, Flores, & Herrero, 2002; Olmedo-Verd *et al.*, 2006).

### 4.3. Other Regulators Co-operating in Gene Activation

Besides NtcA and HetR, other regulators have been identified that have a positive role on gene activation during heterocyst differentiation although their spectrum of action is less extensive and less drastic than that of the principal regulators NtcA and HetR (see Flores & Herrero, 2010). The *nrrA* gene encodes a response regulator of the OmpR family that is expressed from a single Class II NtcA-dependent promoter early upon combined nitrogen deprivation throughout the filament, although expression at later times becomes higher in differentiating cells (Ehira & Ohmori, 2006a; Muro-Pastor *et al.*, 2006). Inactivation of *nrrA* leads to delayed heterocyst differentiation and impaired activation of many nitrogen-regulated genes, studied globally by microarray analysis in an *nrrA* mutant versus the wild-type *Anabaena* sp. strain PCC 7120 (Ehira & Ohmori, 2006a). A direct effect of NrrA on induction of the *glgP1* gene involved in glycogen catabolism and *sigE*, a group 2 sigma factor (see below in this section), upon

nitrogen deprivation has also been described (Ehira & Ohmori, 2011). These results are consistent with the idea that NrrA has a role in the general response to nitrogen deprivation. Because the expression of *hetR* is impaired in the *nrrA* mutant, and because purified NrrA protein binds in vitro to sequences upstream the  $-728/-696$  TSPs of *hetR*, it has been proposed that the NtcA effect on activation of *hetR* transcription from the  $-728$  TSP takes place indirectly through NrrA (Ehira & Ohmori, 2006b).

The *Anabaena* sp. strain PCC 7120 *pipX* gene, encoding a 92-amino acid protein, is activated in cells differentiating into heterocysts, at intermediate-to-late stages of the process, and its inactivation leads to a low nitrogenase activity and impaired diazotrophic growth, which result from the impaired expression of late heterocyst genes (e.g. the *cox2*, *cox3* and *nif-HDK* operons) (Valladares et al., 2011). Taking into account the report of the crystal structure of PipX from *Synechococcus elongatus* in complex with NtcA, consisting of one NtcA dimer and two PipX monomers (Llácer et al., 2010), *Anabaena* PipX may be a co-activator of NtcA reinforcing NtcA-dependent activation of gene expression specifically during the late steps of heterocyst differentiation.

The *hetP* gene encodes a product without recognizable homology and its expression increases localized to proheterocysts (Higa & Callahan, 2010). Ectopic overexpression of this gene leads to some degree of heterocyst differentiation in the absence of a functional *hetR* gene, suggesting a direct function of HetP downstream of HetR. Other regulatory elements, including response regulators, histidine kinases, serine/threonine kinases, HstK kinases (proteins with both a serine/threonine kinase domain and a histidine kinase domain) and CRP homologues, participate in specific steps of heterocyst maturation such as the synthesis and deposition of the Hgl and Hep layers of the heterocyst envelope (Flores & Herrero, 2010; Kumar, Mella-Herrera, & Golden, 2010).

In *Anabaena* sp. strain PCC 7120, NtcA has been shown to activate transcription in vitro promoted by an RNAP including the principal sigma factor, SigA, both at Class II activated promoters (Valladares et al., 2008) and at one HetR-regulated promoter (Camargo et al., 2012). However, this cyanobacterium bears, besides *sigA*, other eleven putative RNAP sigma factor-encoding genes (Aldea, Mella-Herrera, & Golden, 2007). Of these, two group 2 sigma factor genes, *sigC* and *sigE*, and one group 4 gene, *sigG*, are upregulated in differentiating cells at 4 h, 16 h and 9 h, respectively, after nitrogen stepdown (Aldea et al., 2007), and inactivation of *sigE* results in delayed heterocyst differentiation and impaired expression of *nifH* (Mella-Herrera, Neunuebel,

Kumar, Saha, & Golden 2011). However, consistent with its regulation by NrrA (Ehira & Ohmori, 2011), *sigE* could also have a function in vegetative cells in the absence of combined nitrogen. Further studies, which should overcome the complications derived from functional redundancy, will be needed to understand the role played by different sigma factors in the regulation of heterocyst differentiation.

#### 4.4. Regulators Impacting the Pattern of Heterocyst Distribution

##### 4.4.1. *PatS* and *HetN*

As mentioned above, heterocysts are nonrandomly distributed in the cyanobacterial filament and follow a periodic pattern that in strains of the genera *Anabaena* and *Nostoc* consists of a succession of heterocysts separated by c. 10–15 vegetative cells. Several regulators have been identified as involved in heterocyst pattern formation. The *patS* gene is activated early during heterocyst differentiation, and its inactivation produces a ‘multiple contiguous heterocysts’ (Mch) phenotype, whereas its overexpression abolishes differentiation (Yoon & Golden, 1998). This gene encodes a polypeptide whose C terminus consists of the pentapeptide RGSGR, known as PatS-5, which when added to the external medium inhibits differentiation but fails to restore a normal heterocyst pattern in a *patS* mutant (Yoon & Golden, 1998). It has been suggested that PatS, or a derivative of it, is exported from the differentiating cells to build a gradient of an inhibitory signal that prevents the differentiation of its neighbours. Genetic evidence (Khudyakov & Golden, 2004) and in vitro studies (Du *et al.*, 2012; Feldmann *et al.*, 2011, 2012; Huang *et al.*, 2004; Risser & Callahan, 2007) have shown interaction of PatS (or PatS subsets) with HetR, resulting in inhibition of HetR binding to DNA. In *patS* mutants, the Mch phenotype is seen in the first round of heterocyst differentiation after nitrogen stepdown, but it is alleviated later, consistent with a decrease in the expression of this gene.

The *hetN* gene, whose product has putative ketoacyl reductase motifs and includes the RGSGR (PatS-5) pentapeptide, is activated late during differentiation and, as is the case for *patS*, its inactivation produces a Mch phenotype, although this phenotype is expressed at later times than in *patS* mutants. Overexpression of *hetN* also suppresses heterocyst differentiation (Black & Wolk, 1994; Callahan & Buikema, 2001). Whereas conflicting results have been published concerning the role of the reductase activity, the RGSGR sequence appears to be required for the negative effect of HetN on heterocyst differentiation (Higa *et al.*, 2012; Liu & Chen, 2009). Thus,

PatS and HetN appear to act consecutively at establishing and maintenance of the spatial pattern of heterocyst distribution after perception of N stress. A gradient of HetR, dependent on PatS and HetN, has been estimated to take place in the filaments with concentration decreasing in proximity to the heterocysts, and it has been proposed that diffusion of PatS- and HetN-derived signals promote HetR degradation close to the (pro)heterocysts (Risser & Callahan, 2009). It is conceivable that PatS (or a derivative of PatS and HetN) binds HetR inhibiting HetR interaction with DNA and marking it for degradation, with the effect of hampering differentiation of (pro) heterocyst neighboring cells. Nonetheless, the expression of *hetR* is well documented to increase in the cells differentiating into heterocysts (Black, Cai, & Wolk, 1993; Toyoshima et al., 2010). Molecular details of PatS and HetN processing and intercellular transfer, as well as of the HetR post-translational regulation, are missing.

#### **4.4.2. Other elements influencing heterocyst distribution**

The *patA* gene encodes a protein with a C-terminal CheY-like phospho-acceptor domain and an N-terminal PATAN domain possibly involved in protein–protein interactions (Liang, Scarpino, & Haselkorn, 1992; Makarova, Koonin, Haselkorn, & Galperin, 2006), and *patL* encodes a pentapeptide repeat protein (Liu & Wolk, 2011). Inactivation of any of them produces heterocysts mostly located at the filament ends, and PatA and PatL may interact with each other (Liu & Wolk, 2011). In the case of *patA*, this phenotype has been reported to be abolished by inactivation of *patS* and *hetN*, thus implicating PatA in attenuation of the negative signals derived first from PatS and later from HetN (Orozco, Risser, & Callahan, 2006).

The *hefF* gene encodes a putative cysteine-dependent protease, and its mutants show aberrant cell morphology, lack heterocysts and exhibit increased levels of the HetR protein both in the presence and absence of combined nitrogen, but decreased activation of some tested HetR-dependent genes or promoters (Risser & Callahan, 2008; Wong & Meeks, 2001). Overexpression of *hetF* produces an Mch phenotype and induction of the *hetR* gene in all cells of the filament, and it compensates the effect of deletion of the *patA* gene (Risser & Callahan, 2008; Wong & Meeks, 2001). Thus HetF, on which PatA may have a positive effect, appears to be involved in regulation of the HetR levels, both in vegetative cells and heterocysts, as well as of its transcriptional activity (Risser & Callahan, 2008; Wong & Meeks, 2001). However, HetF would not be required for establishment of the HetR gradients promoted by PatS and HetN signals (Risser & Callahan, 2009).

This again stresses the importance of deciphering the post-translational regulation of HetR for the understanding of molecular mechanisms supporting heterocyst differentiation.

The  $\text{Ca}^{2+}$ -binding protein CcbP has been shown to be required for formation of a normal pattern of heterocysts in *Anabaena* sp. strain PCC 7120 (Zhao *et al.*, 2005). An *Anabaena* mutant lacking CcbP shows an Mch phenotype, and a mutant overexpressing CcbP does not form heterocysts. It has been suggested that CcbP sequesters  $\text{Ca}^{2+}$ , which would be liberated upon CcbP degradation in cells that are differentiating into heterocysts (Shi, Zhao, Zhang, Ye, & Zhao, 2006), and this effect has been related to the observation that nitrogen deficiency elicits an increase in the cellular levels of calcium (Torrecilla, Leganés, Bonilla, & Fernández-Piñas, 2004).

The *patU3* gene is expressed in proheterocysts at late differentiation times, and its inactivation produces an Mch phenotype (Zhang *et al.*, 2007). The *hetZ* gene, upregulated in proheterocysts, encodes a putative DNA-binding protein with a helix–turn–helix motif, and its inactivation provokes delayed or no differentiation while impairing the induction of some heterocyst differentiation genes such as *patS*, *hetC* and *cox2*, and altering the patterned induction of *hetR* (Zhang *et al.*, 2007). Thus, PatU3 and HetZ have been proposed to influence the co-ordination between heterocyst differentiation and pattern formation (Zhang *et al.*, 2007; see also Meeks *et al.*, 2002).



## 5. CONCLUSIONS AND PERSPECTIVES

We have described above the patterns of regulation of different types of genes that are expressed during the process of heterocyst differentiation, and have summarized some aspects of their regulation. A salient feature is the conspicuous role that the NtcA transcription factor plays at the beginning of differentiation, during the differentiation process and in the mature heterocyst (Herrero *et al.*, 2004). The molecular basis for this role of NtcA is the presence of NtcA-dependent promoters in many heterocyst-related genes. But HetR, which can now be denoted a transcription factor as well (Kim *et al.*, 2011), is also needed for activation of transcription of a number of these genes. The mechanism by which HetR exerts its role is unknown, but the first hint is that it could be a co-activator of transcription at some NtcA-dependent promoters (Camargo *et al.*, 2012), consistent with the fact that all HetR-dependent promoters are also dependent on NtcA (although indirect effects of NtcA cannot be ruled out). As is the case

for NtcA, which has 2-oxoglutarate as an effector, HetR could be subjected to post-translational regulation, a possibility that will merit further research. Other co-activators of NtcA-dependent transcription appear to exist, as is the case of PipX (Valladares et al., 2011), asking whether a very elaborated transcriptional complex, perhaps including alternative RNAP sigma factors (Aldea et al., 2007), might be acting in transcription of heterocyst-related genes.

Consistent with a specific function of their protein products during the differentiation process, a number of heterocyst differentiation genes are only transiently expressed after combined-nitrogen deprivation. However, the mechanism of downregulation of such genes is unknown. In this context, the possible role of N<sub>2</sub>-fixation products once heterocyst differentiation is completed will merit further investigation. Related to this question is that of regulation during established diazotrophic growth. The cells in the filament grow and divide, and when the heterocysts are separated by too many vegetative cells, a cell in between differentiates into a new heterocyst. The basis for this differentiation process might be different from that of the synchronous heterocyst differentiation that takes place in filaments subjected to nitrogen stepdown, but possible mechanisms of regulation have been less extensively investigated. Availability of nitrogen, which might be scarce for vegetative cells away from a heterocyst, might again have a role (Wolk & Quine, 1975; Yoon & Golden, 2001). Thus, the study of gradients of metabolites that might influence gene expression will also be of much interest.

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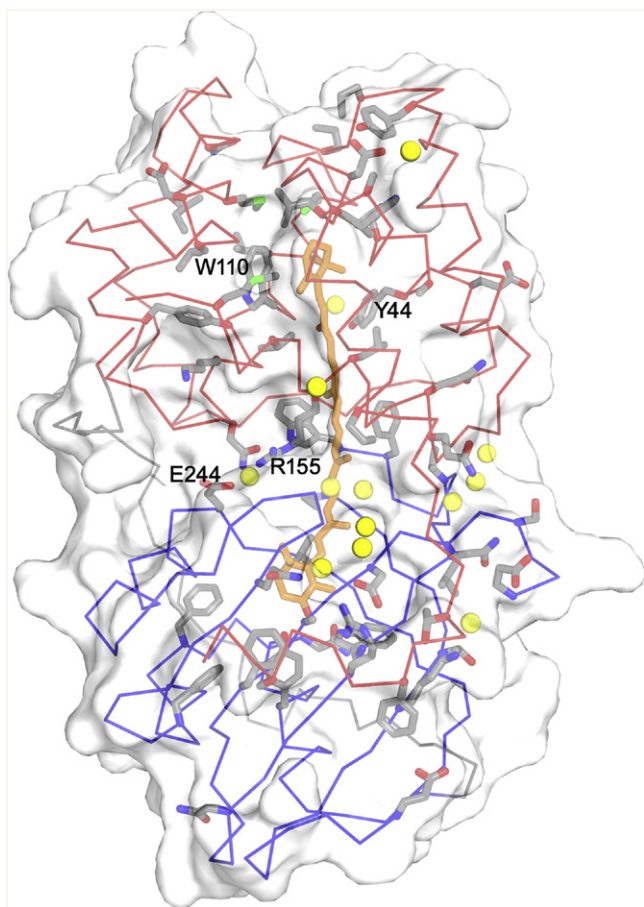
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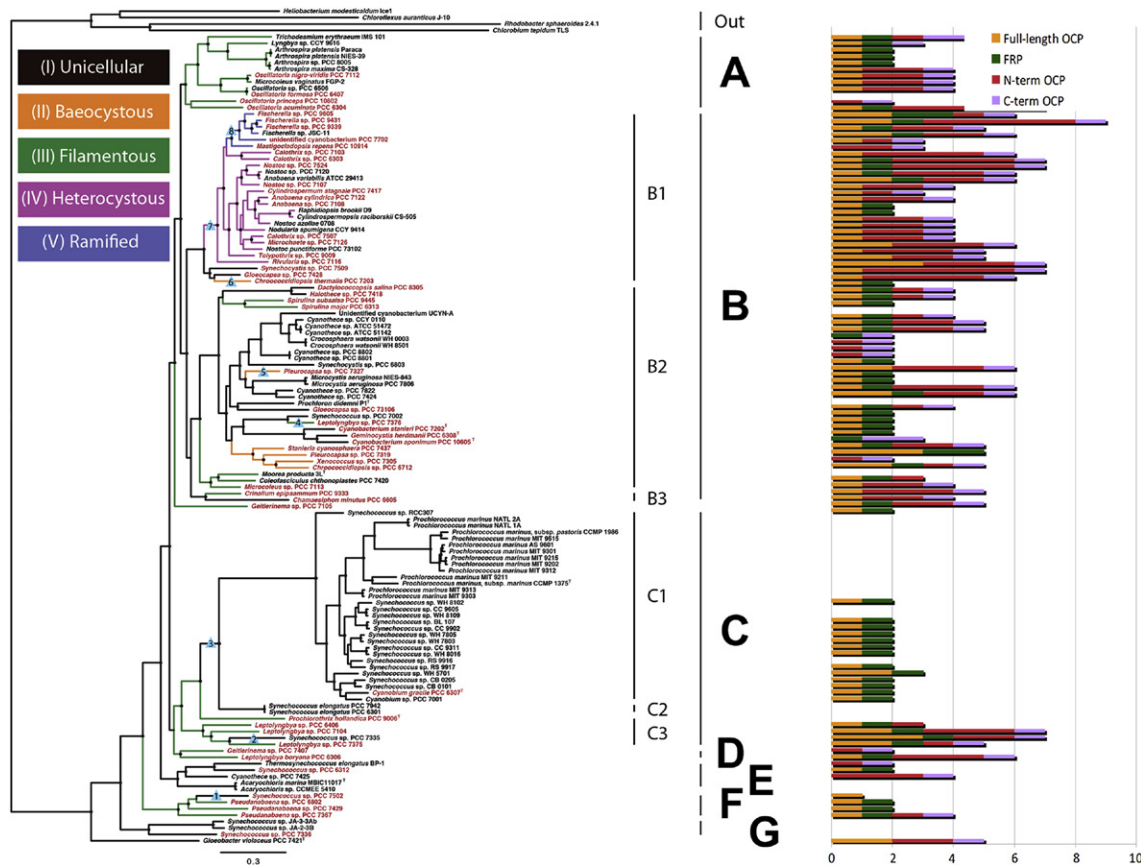
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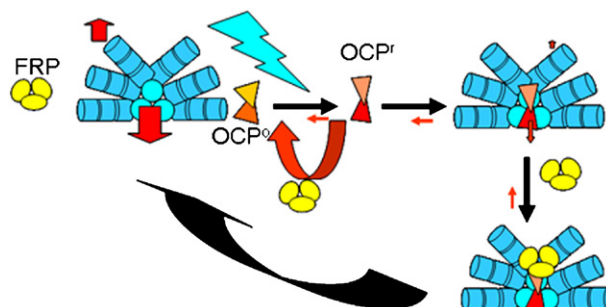
## COLOR PLATES



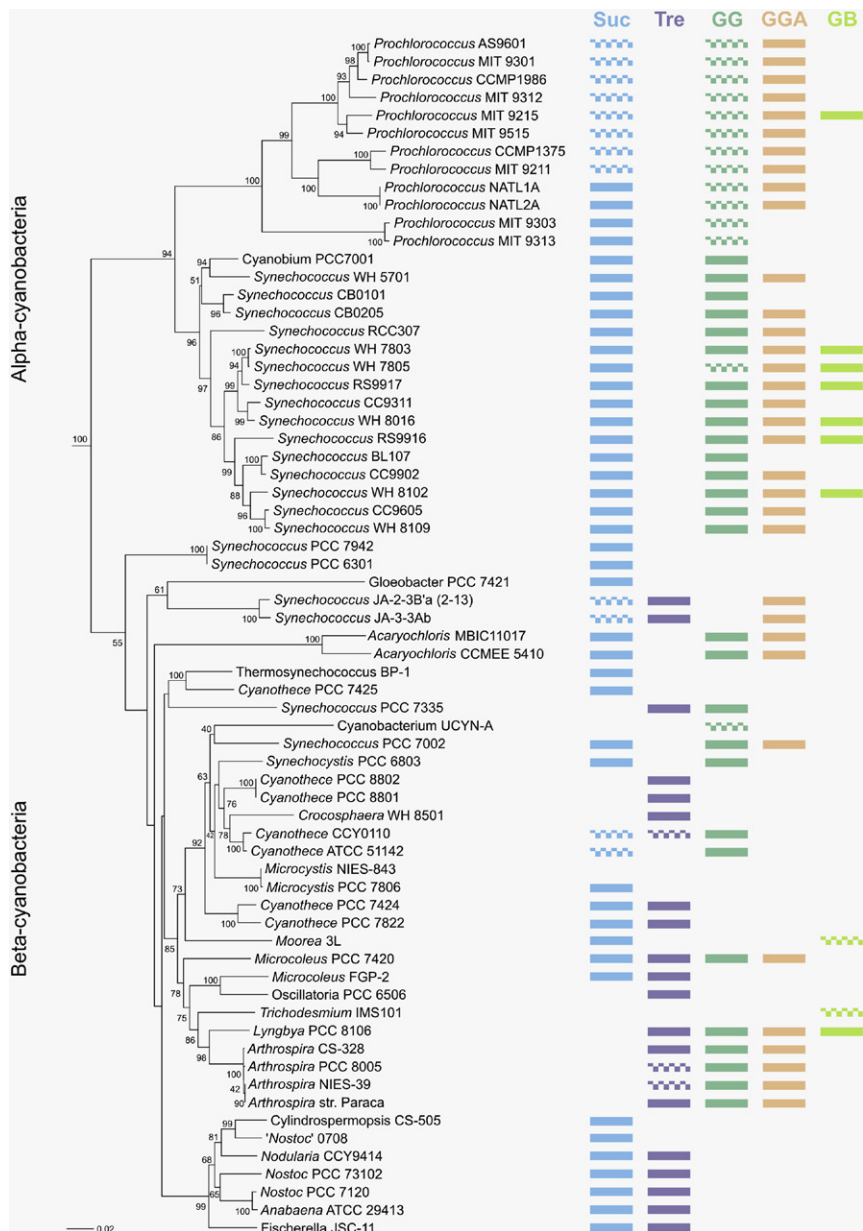
**Figure 1.1** The structure of the OCP. The N-terminal domain is uppermost, with the backbone traced in red; the C-terminal domain is traced in red. The carotenoid is shown in sticks as are all absolutely conserved amino acids among the 90 currently available OCP sequences. Conserved water molecules are shown as spheres. Figure made with pymol (<http://www.pymol.org/>).



**Figure 1.3** Current census of the distribution of genes encoding the OCP, FRP and genes for the N- and C-terminal domains of the OCP, based on sequence data described in Shih et al., (in press). Clades and subclades assigned are marked with letters.

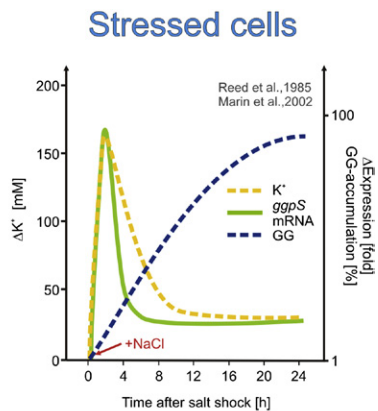
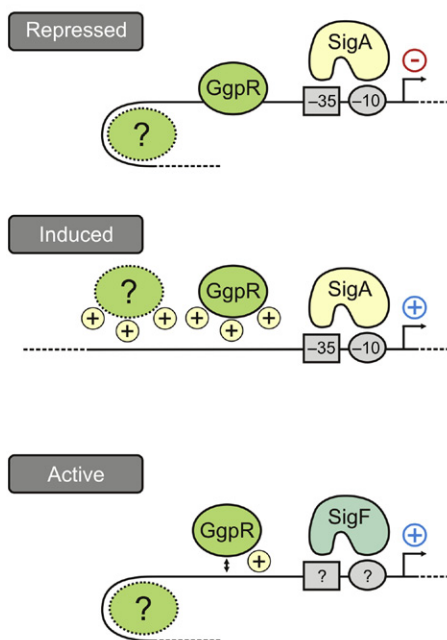


**Figure 1.4** Schematic of the current understanding of the OCP photoprotective mechanism. Light (thunderbolt) activates the OCP (fused triangles) converting OCP<sup>o</sup> into OCP<sup>r</sup>. Only OCP<sup>r</sup> is able to bind to the core of phycobilisomes or to FRP (ovals, here shown as a trimer). These interactions are light independent. Fluorescence quenching depends on the concentration of OCP<sup>r</sup> and on the FRP/OCP ratio. Vertical arrows symbolize energy flow, either to the reaction centre or dissipated as heat.

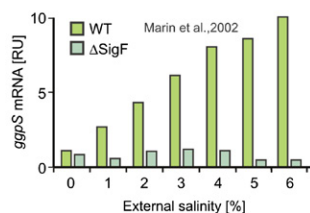


**Figure 2.1** Phylogenetic tree (neighbour joining algorithm) of 67 cyanobacterial strains with known genome sequence. The tree is divided into the large groups of alpha- and beta-cyanobacteria. The colour bars represent whether or not genes for compatible solute biosynthesis (Suc–sucrose; Tre–trehalose; GG–glucosylglycerol; GGA–glucosylglycerate; GB–glycine betaine) were found in the genome sequences (see Table 2.1). Dotted bars indicate that the pathway is only incomplete.





### Long-term acclimation



**Figure 2.2** Model of transcriptional regulation of the *ggpS* gene for glucosylglycerol (GG) synthesis (left panels) in *Synechocystis* 6803 in comparison to experimental data (right panels). In low-salt grown cells, the *ggpS* gene is repressed leading to GG-free cells. Salt-shock treatments induce the highest *ggpS* expression and quick GG accumulation because the influx of inorganic ions releases the repressor GgpR from the *ggpS* promoter in salt-stressed cells. In long-term salt-acclimated cells, the *ggpS* expression remains active but depends on the external salinity. Moreover, the *ggpS* expression in salt-acclimated cells seems to be driven by SigF instead of SigA because a SigF deletion abolishes *ggpS* expression to a large extent. Possibly, additional regulatory factors (marked by ?) are also involved.

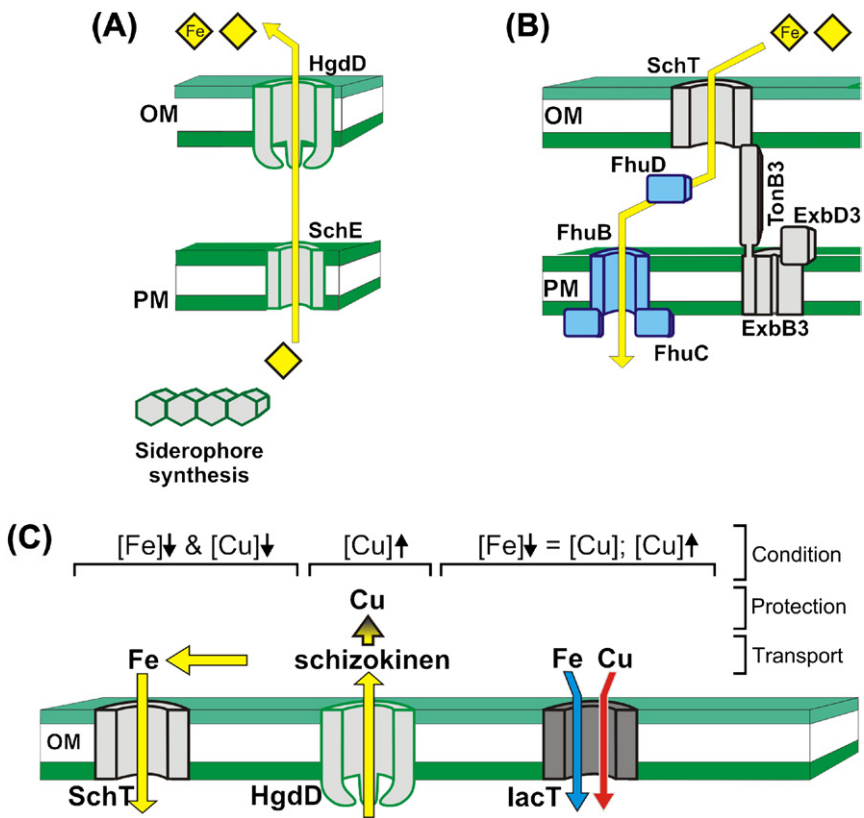


Figure 3.4 Iron uptake pathway in *Anabaena* sp. PCC 7120.

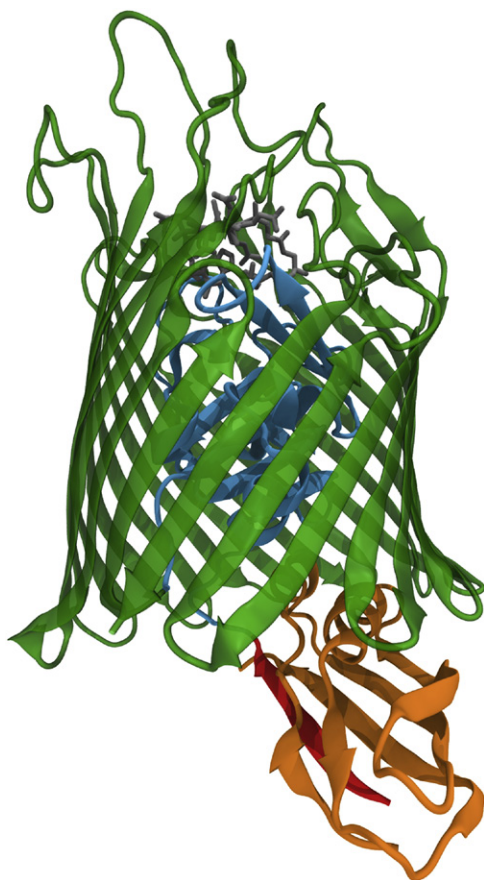
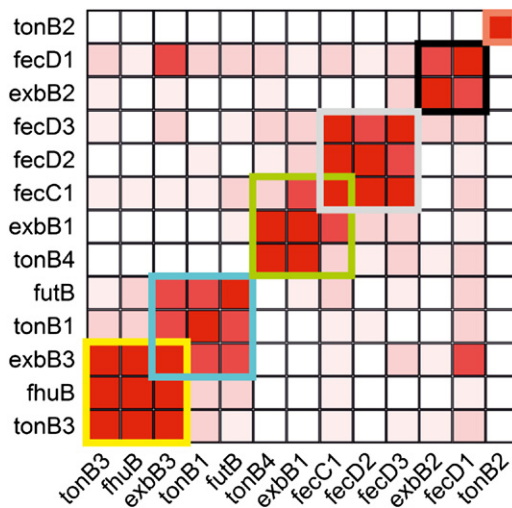
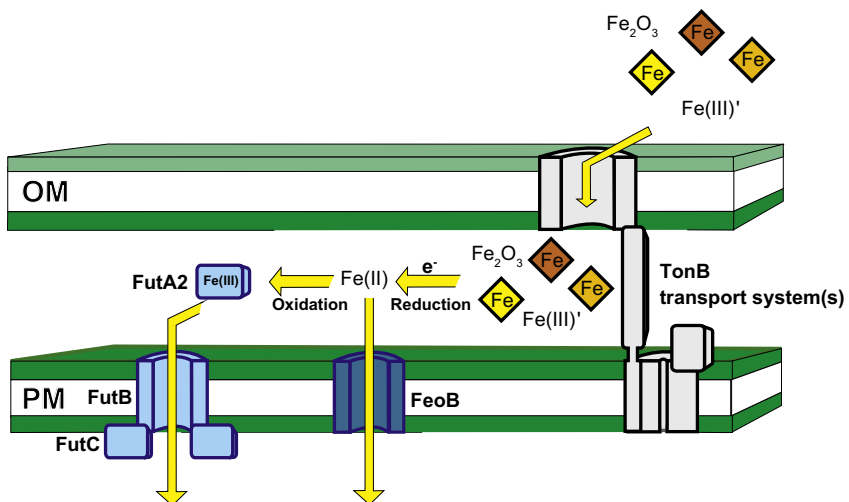


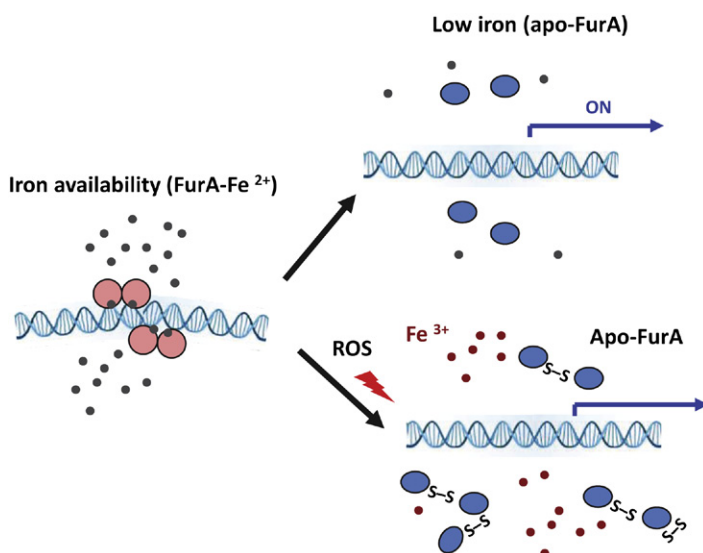
Figure 3.5 Stricture of a TBdT.



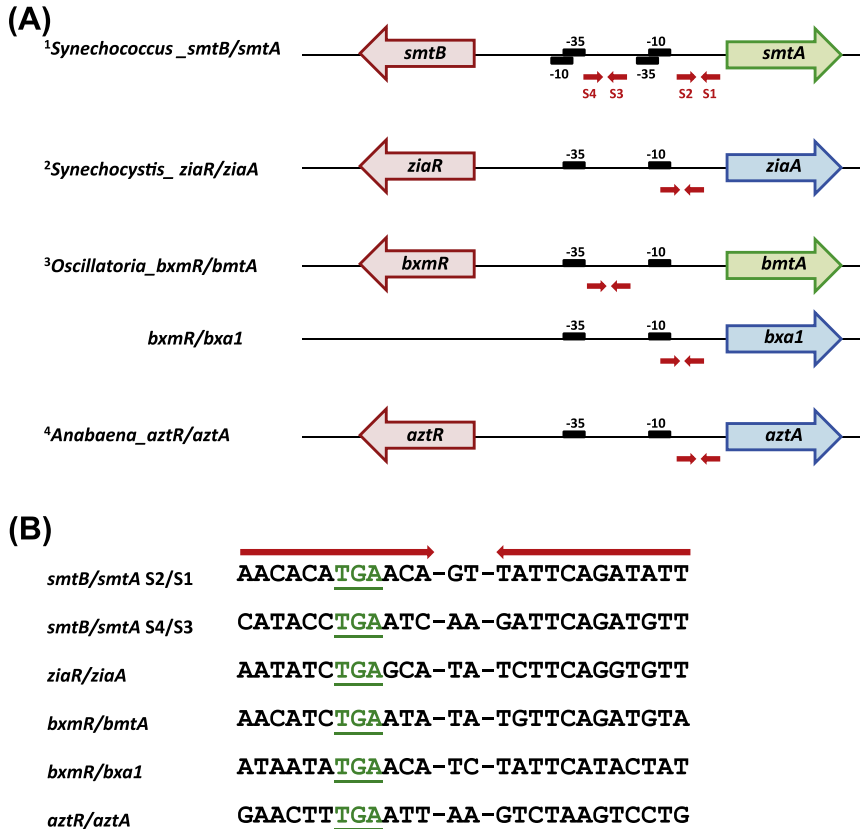
**Figure 3.6** The correlation of the expression profiles for the different iron transport components.



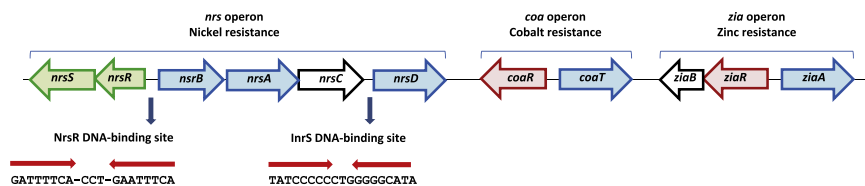
**Figure 3.7** Uptake pathway model for *Synechocystis* PCC 6803.



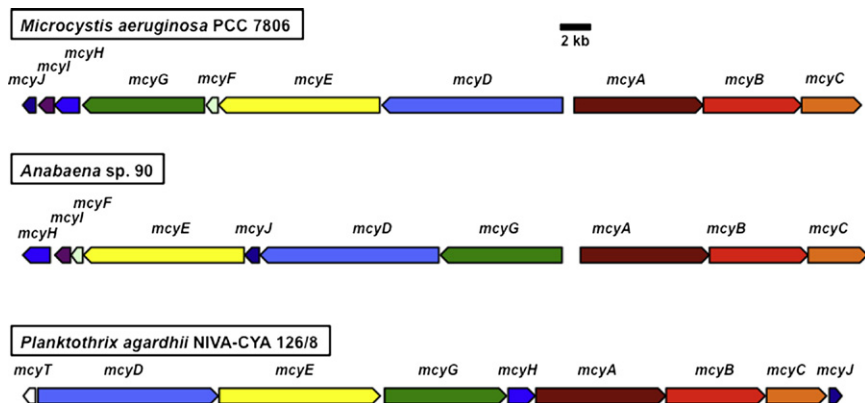
**Figure 4.3** Proposed model for the influence of the intracellular iron concentration and the presence of reactive oxygen species (ROS) in FurA activity.



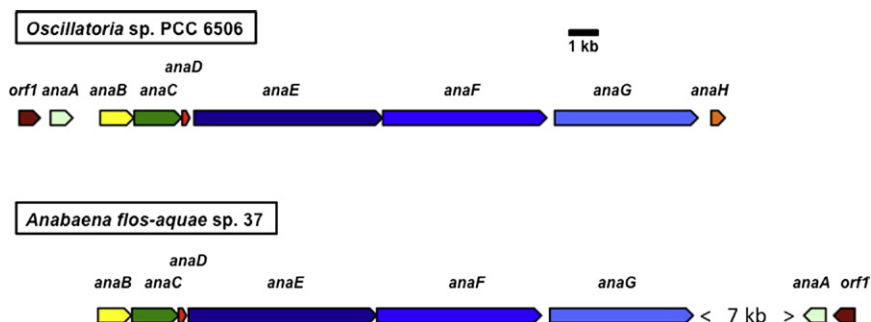
**Figure 4.6** A. Organization of the operons encoding cyanobacterial metal-regulated SmtB repressors. Genes encoding metalloregulators (dotted arrows), ATPase genes (grey arrows) and genes encoding metallothioneins (white arrows) are represented. Black arrows indicate the imperfect inverted repeats where the SmtB regulators are bound. (<sup>1</sup>(Erbe et al., 1995); <sup>2</sup>(Thelwell et al., 1998); <sup>3</sup>(Liu et al., 2004); <sup>4</sup>(Liu et al., 2005)). B. Alignment of the DNA-binding sites containing the 12-2-12 inverted repeat sequences from the operons controlled by cyanobacterial SmtB metalloregulators. The conserved sequence TGA which is supposed to be in contact to the regulators is underlined.



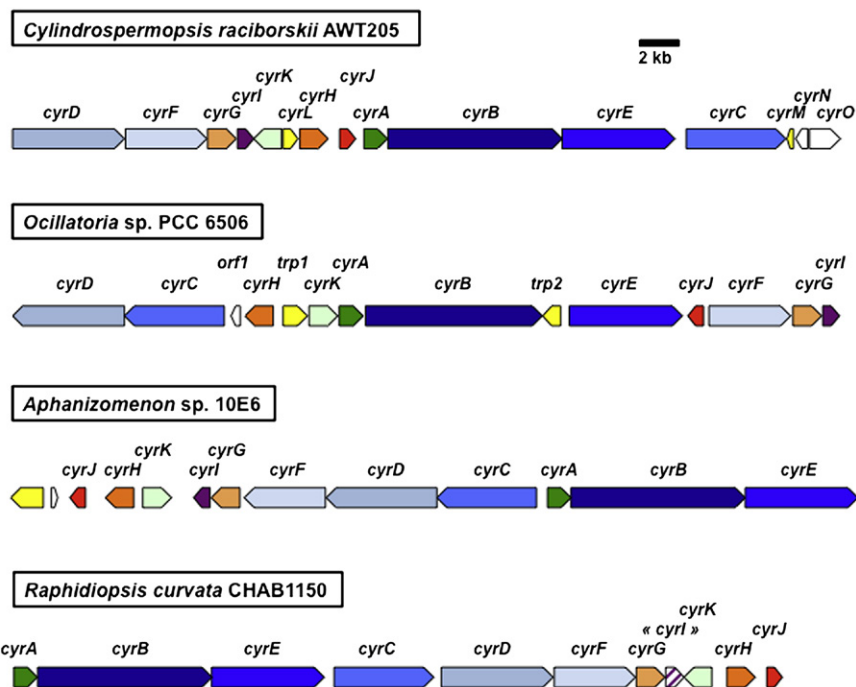
**Figure 4.7** Genetic organization of the metal-regulated cluster in *Synechocystis*. The different metal-resistance operons and target DNA sequences for nickel resistance are indicated. Genes encoding metalloregulators (dotted arrows), ATPase genes (light grey arrows), genes encoding the two-component system NrsRS (dark grey arrows) and genes with unknown function (white arrows) represented. Direct or inverted repeat sequences present in the NrsR and InrS DNA-binding sites are denoted with arrows (Foster et al., 2012).



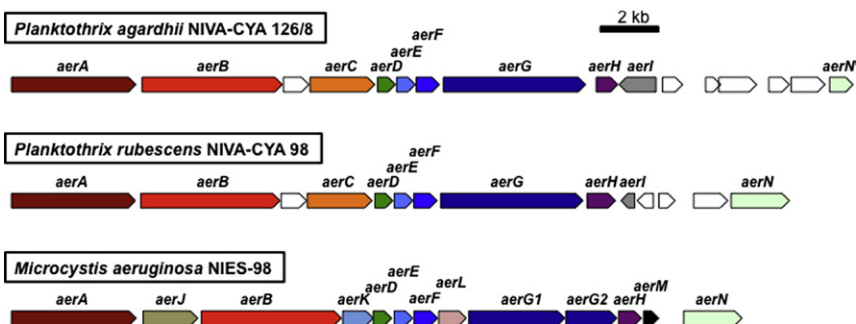
**Figure 6.2** Comparison of three selected *mcy* gene clusters from different genera. Note that the *Planktothrix agardhii* *mcy* cluster lacks the *mcyI* and *mcyF* genes (implicated in the biosynthesis of erythro-2-methyl-D-Asp) and has a supplementary type-II thioesterase gene, *mcyT*.



**Figure 6.5** The two *ana* gene clusters so far identified.

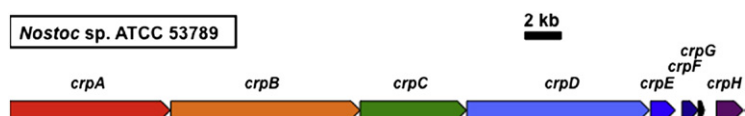


**Figure 6.8** The four *cyr* clusters so far identified. The transposase genes are coloured in yellow. The *cyrN* and *cyrM* genes in *Cy lindropermopsis raciborskii* were not found in the other *cyr* clusters. In *Raphidiopsis curvata*, *cyrI* is a pseudogene, and this strain produces 7-deoxycy lindropermopsin.

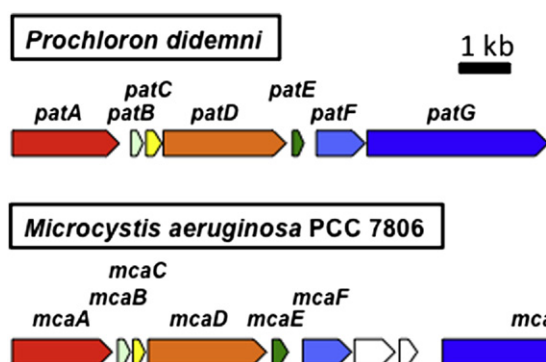


**Figure 6.11** Selected *aer* gene clusters identified in different cyanobacteria.

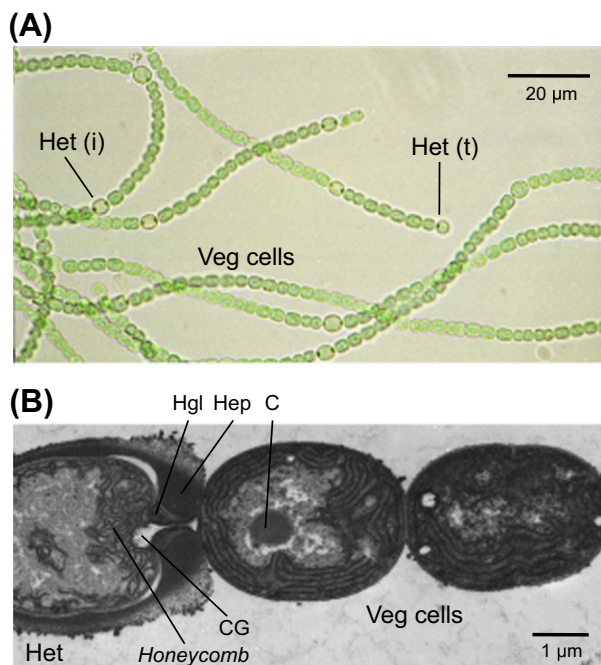




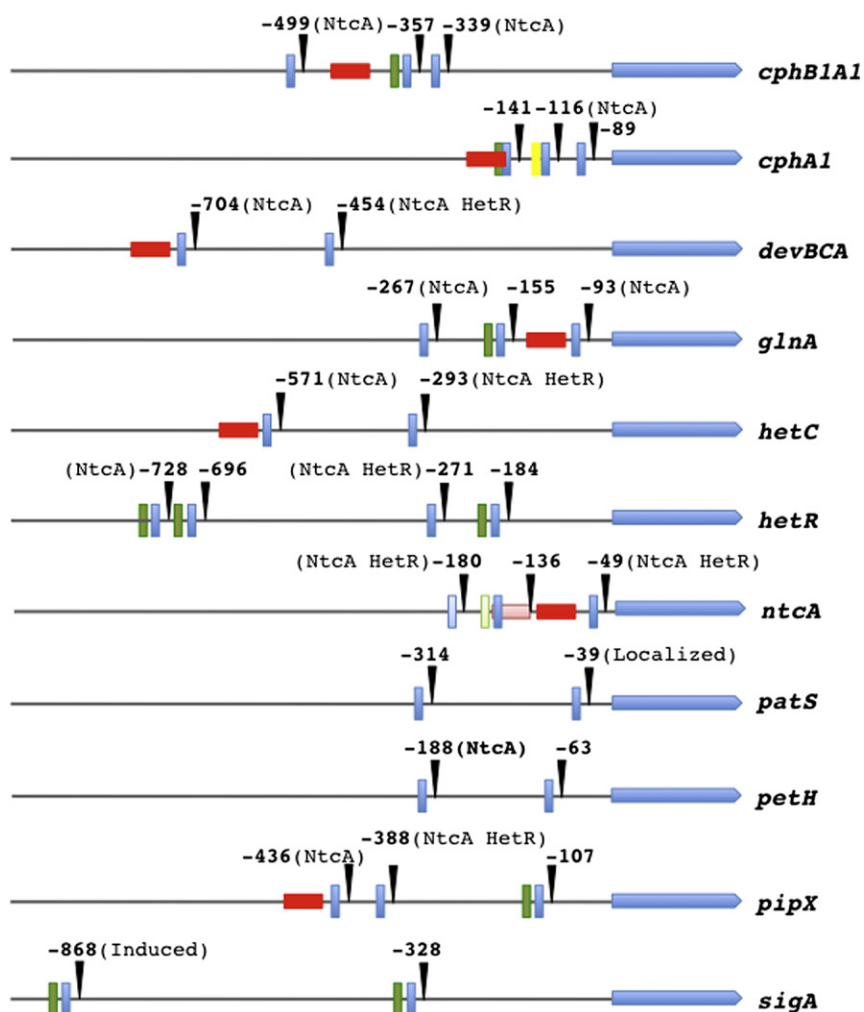
**Figure 6.14** The gene cluster *crp* responsible for the biosynthesis of cryptophycin 1 in *Nostoc punctiforme* ATCC 53789.



**Figure 6.16** Comparison of the cyanobactin clusters responsible for the biosynthesis of patellamide and microcyclamide.



**Figure 8.1** Parts of filaments of *Anabaena* sp. strain PCC 7120 observed by light microscopy (A) or transmission electron microscopy after chemical fixation (B), which was performed as described by Merino-Puerto et al. (2011). Het, heterocyst, which may be placed intercalary (i) or terminally (t) in the filament; Veg cells, vegetative cells; Hgl, heterocyst glycolipid layer; Hep, heterocyst envelope polysaccharide layer; C, carboxysome; *honeycomb*, heterocyst intracellular membrane system; CG, place where the cyanophycin granule (lost during sample preparation) was located.



**Figure 8.2** Complex promoter regions of some *Anabaena* sp. strain PCC 7120 genes participating in heterocyst differentiation or function (Table 8.2). The experimentally determined dependence on NtcA and HetR is indicated. Black triangles indicate TSPs; blue boxes, -10 determinants; green boxes, -35 determinants; yellow box, a putative UP element; red boxes, NtcA-binding sites. Imperfect determinants are indicated with the corresponding light colours.